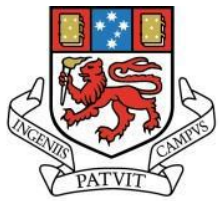

UNIVERSITY
OF TASMANIA

ECTOPARASITES AND ASSOCIATED PATHOGENS AFFECTING FARMED SALMON DURING MARINE GROW OUT IN CHILE AND AUSTRALIA

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ABSTRACT

This research characterized some outbreaks of the ectoparasite *Caligus rogercresseyi* in Chile and compared them to similar infestations in Australia by *Caligus longirostris* and *Ceratothoa banksii*, in relation to co-infections by piscirickettsiosis and viral infection salmon anaemia (ISA virus (ISAV)) in Chile and/or amoebic gill disease (AGD – both in Australia and Chile), and according to husbandry management practices. Understanding how and why co-infections occur allows the development of preventive integrated strategies. The co-infection of these pathogens could occur because 1) salmon, normally resistant to a pathogen become, more susceptible to it, due to pre-infection with an ectoparasite or vice-versa, and 2) salmon farms have single-pathogen oriented control strategies that are ineffective.

To identify the reservoirs for *Neoparamoeba perurans* (AGD etiological agent) on ectoparasites from farmed salmon in Tasmania, the presence of this amoeba was investigated on the gill isopod *Ceratothoa banksii* collected from farmed salmon. Using quantitative real-time PCR analysis it was demonstrated that *N. perurans* was either present in low concentration or absent on the surface, and absent inside the gill isopods from salmon that had not been freshwater bathed for AGD during 200 or more days. Thus it is unlikely that these isopods acted as reservoirs or vectors for the amoeba. In contrast, with the potential advent of alternative (non-freshwater) AGD treatments it is possible that Ceratothoid parasitism will become a potential problem on Tasmanian salmon farms.

An investigation of the environmental reservoirs of *N. perurans* during tank-based experimental infections demonstrated that the concentration of *N. perurans* was significantly higher in the running water (13 ± 7 cells/L after 16 days) than on the interphase surface air-water-tank (0.01 ±0.1 cells/L). Although the number of *N. perurans* on fish gills was not determined in this tank-based study, on farmed Atlantic salmon from the Huon estuary chronically affected with AGD, amoebae detected from gill swabs ranged from 0-1042 cells/swab, which could indicate that fish themselves are reservoirs of this pathogen.

To characterize the outbreaks of the sea lice with the other main pathogens (*N. perurans, Piscirickettsia salmonis* and ISAV), moribund salmonids were sampled in Chile in 2011 from two neighboring farms. On one farm, rainbow trout were bath-treated with deltamethrin for sea lice fortnightly in October and November and Atlantic salmon from the other farm treated only

after the monitoring on 9 November. Co-infection with *P. salmonis* was present in 28% of rainbow trout and 10-12% of Atlantic salmon, with asymptomatic ISAV (pathogenic strain HPR 7b) in 17% and 80% of the trout and salmon respectively. Fish had not been treated for sea lice despite having a parasite load exceeding 6 lice per fish which is the load where treatment is mandatory. They also showed microscopically and gross gill damage or excess mucus production. The etiological agent of this gill pathology is still unclear, as fish and sea water from farms were free from *N. perurans.* Water samples did not contain any significant levels of contaminants, diatoms or dinoflagellates and gill damage was not caused by the chemical baths. Apparently healthy Atlantic salmon sampled in 2013 in a farm in Chile that had been subjected to monthly cypermethrin lice treatments, showed no *P. salmonis* but 33% of the fish in April and 22% in June, were asymptomatic carriers of the pathogenic strain ISAV (HPR 7b). The routine monitoring mortalities in this farm determined that 2% and 12% died by piscirickttsiosis in April and June respectively. Sea lice abundance was lower in most netpens in April with higher water temperatures and AGD presence compared to June with lower temperatures and without AGD. A diverse level of chronic gill lesions were observed on fish samples such as the undetermined gill pathology on fish in 2011, seasonal AGD in March-April 2013 and multiple mucous cells in June 2013. This gill pathology could be the source of fish mortality during sea louse treatments. It could also cause stress on farmed fish that can promote the fast multiplication of the remaining sea lice on the immunosuppressed farmed fish. This could effectively maintain a chronic infestation. Systematic chemical baths that are compulsory for sea lice treatment in Chile can increase gill damage, they can cause stress and predispose fish to outbreaks of ISA or piscirickettsiosis. A reduction of frequency of chemical baths is advised, particularly considering that in Chile, there are no wild populations of fish that need protection from *C. rogercresseyi* released from farms. This will also help with the fulfilment of the good practice measures to control sea lice and ISA by the reduction of salmon handling and limited use of chemical therapies. In addition, on farms with piscirickettsiosis outbreaks, the daily removal not only of mortality but particularly of moribund fish is required. In relation to environmental friendly treatments, although freshwater baths for AGD could be an alternative control for sea lice, the location far from estuarine areas of most salmon farms in Chile and the lack of plentiful freshwater indicate difficulties for the implementation of this method.

CHAPTER 1

GENERAL INTRODUCTION

Salmon and trout aquaculture is a growing industry in temperate zones throughout the world (FAO, 2009). Salmon are intensively reared using large initial investments and industrial technology enabling great control and high production. In the production cycle, breeding and maintenance of broodstock and culture of juvenile stages are carried out in fresh waters, in tanks on land-based systems or cages in inland waters; the ongrowing of fish to market size occurs, in open marine net-pens (Jones, 2004). Although aquaculture is the fastest growing animal food-producing industry in the world, important differences can be found in different countries in relation to production volumes and disease outbreaks. As finfish aquaculture develops, diseases affecting reared species continue to emerge (Davenport *et al.*, 2003). Chile is one of the major producers of salmon in the world but experiences the negative impact of diseases (Alvial *et al.*, 2012). The industry in Australia is much smaller and does not have same disease impacts (Battaglene *et al.*, 2008).

Atlantic salmon is a fish species of high commercial value and aquaculture production has overtaken the fishing production (Jones, 2004). This fish species is native to the Atlantic waters in the northern hemisphere around countries such as Scotland, Ireland, Norway, Canada and United States, where wild populations have been declining due to overfishing. In contrast, farmed populations are on the rise in native areas (e.g. northern Europe, eastern North America) as well as in regions where they are introduced (e.g. western North America, Chile, Tasmania) (Battaglene *et al.*, 2008; Naylor *et al.*, 2008). The major producers of Atlantic salmon in 2012 were Norway (1232 MT) and Chile (386 MT), where the fish are reared mostly for the export market, Australia was the sixth in the ranking (43 m t) (FAO, 2014) with most of the production being sold at the domestic market, due to a high global production and transportation cost and lower price in external market (Battaglene *et al.*, 2008). The strategy adopted by the Chilean industry was production of larger volumes, diversifying products and finding new markets (Infante, 2003; Norambuena and González, 2005).

Most of the activity in Chile was concentrated in the South, allowing the development of this area through the generation of local jobs on farms, processing plants and related services (Fig. 1.1). The main number of farms registering salmon harvesting were located in the southern X region (Sernapesca, 2007b), although the hatcheries and farms producing smolts are located in more northern regions, between the V to X regions. However, after disease outbreaks in marine cultures in 2006-2007 some companies moved to the next more southern XI region (Sernapesca, 2009). A minor proportion of farms are located in the southern and colder XII region. The XI region can still allow the expansion of the industry for marine net-pen systems (Norambuena and González, 2005). In relation to the species cultured in Chile, the government reported in 2005 (Subpesca 2005) that most important fish species reared in sea-cages are Atlantic salmon *Salmo salar* (Linnaeus, 1758), coho salmon *Oncorhynchus kisutch* (Walbaum, 1792) and rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) which are all exotics. The composition of cultured salmon species in Chile has been changing during the last decade due diseases. In 2011, the distribution per species of fish stocked in sea water in this country was 58% Atlantic salmon, 24% rainbow trout and 16% coho salmon (Alvial *et al.*, 2012). One possible reason to explain the rise of disease incidence in Chile is the importation of salmon eggs from the northern hemisphere. However, although the total quantity of eggs imported diminished in 2003 due to the sanitary policy established that year, a proportion of eggs (18%) are still imported to provide eggs in periods out of natural seasons (Norambuena and González, 2005; Subpesca, 2008; Alvial *et al.*, 2012).

In the case of Australia, salmon farming is the most valuable national seafood industry but based only in Tasmania (Fig. 1.2), as well as being the major regional and youth employer (Battaglene *et al.*, 2008). The production of this finfish is concentrated in 3 sheltered regions and off the island. Legislation does not allow their rearing in other Australian states, for instance South West Australia where Blue Fin tuna are cultured in sea cages with ranching culture systems (Buckely and Gilligan, 2005; Skonhoft, 2005). Besides, in Tasmania there are no other marine areas which are suitable for salmon culture using current technology (Battaglene *et al.*, 2008). Salmonid farming began in Tasmania as in Chile, through private and government activities with rainbow trout in 1981. Commercial salmon aquaculture started in Tasmania in 1984, thanks to a cooperative project between the state government, the Norwegian company Noraqua and the industry. At that time a large hatchery was established using European technology, good sites and water quality to produce the number of smolts (or

juveniles) required per year to support all national production. Despite salmon aquaculture started with the introduction of eggs, no future importations were allowed to support the development of the industry, contrary to the Chilean industry. Buckley & Gilligan (2005) indicated that the present environmental regulations in Australia, do not allow the import of new eggs and smolts as for other exotic fish species. Imports of any genetic material were banned in the 1960s, to protect against introduction of exotic pathogens. Ninety percent of total salmon marine production is composed of Atlantic salmon, 9% of rainbow trout and 1% of brook trout *Salvelinus fontinalis* (Mitchill, 1814) all introduced only for marine cage culture (Battaglene *et al.*, 2008).

In the production cycle, broodstock of farmed salmon are selected from sea site farms and moved into freshwater tanks or cages for egg stripping, hatching and alevin incubation in silos or trays. Then, fry, parr and finally smolts are produced in tanks or cages (Jones, 2004). Smolted salmon are transferred to sea sites when they are adapted for seawater survival and then transferred by road, helicopter and/or sea in specialized wellboats that are boats with large wells circulating seawater. Most ongrowing at sea takes place in cages consisting of nets suspended from various floating systems anchored to the seabed (Jones, 2004). Most cages in Chile are 30x 30 m square shaped and 15 to 20 m deep, suspended from various floating walkway systems and grouped together in single or double "cages-train". Meanwhile in Australia most cages are larger circular pole cages that can reach 35 m in diameter and are grouped together to form a sea site.

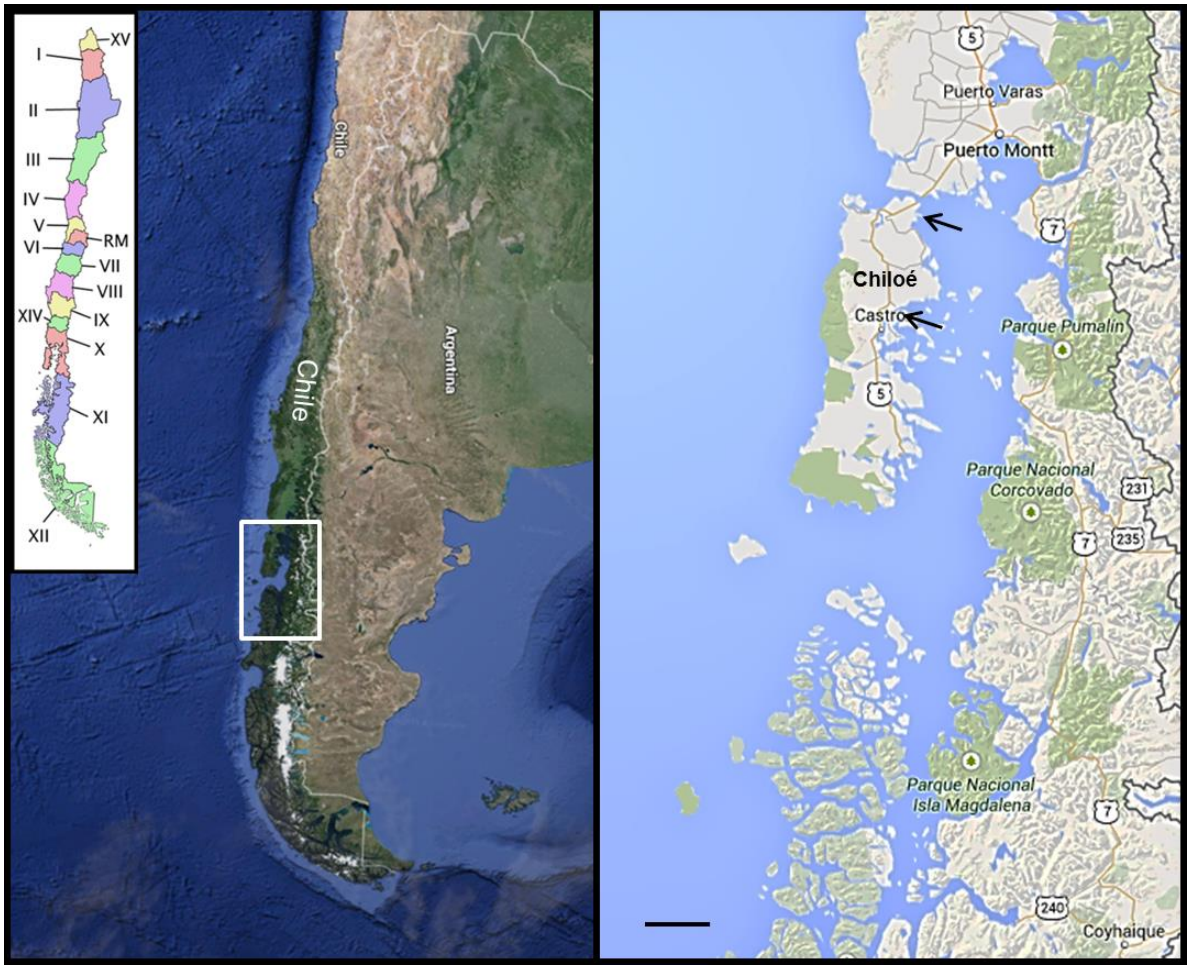


Figure 1.1. Regional map of Chile. Most of salmon farming occurs at the southern inner sea. (Arrows: Farms sampled in this study, Bar: 50 km; Source: Google Map).

Figure 1.2. Tasmania, the most southern state of Australia (Arrows: farms sampled in this study Bar: 20 km; Source: Google Maps).

In Chile, current mitigation strategies for the most important parasites are based on chemotherapies (Roth, 2000; Sernapesca, 2013) that are expensive, often ineffective and at the same time have a detrimental impact on the environment where they are used. In Tasmania, the major disease is amoebic gill disease (AGD) caused by the ectoparasitic amoeba *Neoparamoeba perurans* (Young *et al.*, 2007) (Protozoa:Amoebozoa). The disease severely impacts the salmon industry in Tasmania (Bridle *et al.*, 2010) as do sea lice *Caligus rogercresseyi* (Boxshall and Bravo, 2000) (Crustacea: Copepoda: Caligidae) in Chilean salmon aquaculture (Johnson *et al.*, 2004). Parasites such as isopods (Crustacea) and sea lice are virtually absent from salmon farms in Tasmania where freshwater baths are regularly applied to treat AGD (Nowak *et al.*, 2011). This indicates a possible environmentally friendly alternative of treatment for these ectoparasites in Chile.

1.1 Impact of diseases on salmon aquaculture in Chile compared to Australia

1.1.1 Chile

Since the end of 2006 deterioration in the status of the production parameters of intensive farmed salmon has been observed in the south of Chile. Farmed salmon have shown low food

consumption, high mortalities, poor growth and food conversion factors as well as high infestation with *C. rogercresseyi* (Bustos *et al.*, 2011). Histopathological and molecular studies of affected fish confirmed a diagnosis of AGD which was considered an emerging parasitic disease in Chile. Amoebic gill disease was first identified in Washington State in 1985 (Kent *et al.*, 1988), in Australia in 1986 and later diagnosed in Atlantic salmon in several countries (Munday *et al.*, 2001; Young *et al.*, 2008b; Crosbie *et al.*, 2010). The disease had already been reported in Chile in 1990 (Nowak *et al.*, 2002) from salmonids affected by piscirickettsiosis and then in 2007 from salmonids affected with sea lice (Rozas *et al.*, 2012), but its significance to salmon production was not well understood

In 2007 an emergency was declared in Chile due to an outbreak of infection with *Caligus rogercresseyi* (Sernapesca, 2007b; 2008; Hamilton-West *et al.*, 2012). Despite the joint efforts of the government, private industry and universities in managing this sanitary problem since 1994, solutions are still far from being reached even though many years of work and large amount of funds have been invested in sea lice research (Reyes and Bravo, 1983; González and Carvajal, 1994; González *et al.*, 1997; Carvajal *et al.*, 1998; González and Carvajal, 1999; Hidalgo and Cassigoli, 1999; Boxshall and Bravo, 2000; Carvajal and Sepúlveda, 2002; González and Carvajal, 2003; Johnson *et al.*, 2004; Sepúlveda *et al.*, 2004; González, 2006; Marín *et al.*, 2007; Rozas and Asencio, 2007). As a practical strategy the integrated *Caligus* Monitoring and Control Program from INTESAL was implemented since 1999 to 2006 (Zagmutt-Vergara *et al.*, 2005), which was followed by the National Monitoring and Control Program implemented by Sernapesca in 2007 due to new surges in sea lice (Molinet *et al.*, 2011; Yatabe *et al.*, 2011; Hamilton-West *et al.*, 2012). Treatments with a new in-feed chemical, emamectin benzoate (Schering-Plough, 2005), were applied between 2000 and 2007 avoiding the problematic use of chemical baths and allowing the simultaneous treatment of whole farms. Nevertheless, soon after application of the treatments reduced sensitivity of *Caligus* to this chemical was reported (Bravo *et al.*, 2008a; Jones *et al.*, 2013; Bravo *et al.*, 2014). As alternative treatments, several previously used chemical compounds were approved and implemented with regulations, together with an integrated management strategy by the also regulated Sanitary Areas (Yatabe *et al.*, 2011; Sernapesca, 2012; 2013). However, despite these measures the sea lice outbreaks have not stopped (Yatabe *et al.*, 2011; Bravo *et al.*, 2013).

In mid-2007, an outbreak of the viral disease infectious salmon anemia (ISA) added another problem to the sanitary situation in Chile. This virus is classified in the new genus *Isavirus* and had been associated with high accumulated mortality of farmed Atlantic salmon exceeding in severe cases 90% mortalities in 3 months (OIE, 2013). As contingency measures in 2008, infected farms were forced to harvest their fish and close for periods of 3 months or more to allow fallowing. This situation led to a drop in the production of some farms, high unemployment levels and the expansion of salmon aquaculture from one region (Region X) to two additional regions (Region XI and XII). Although this procedure reduced the farm densities, it did not reduce the incidence of the disease as expected and caused spreading of ISA from the focus area to the other two areas (Sernapesca, 2010).

1.1.2. Australia

Amoebic gill disease is the major disease impacting Atlantic salmon production in Tasmania and its incidence and prevalence has grown in line with an increase of salmon production (Crosbie *et al.*, 2005) and so have the costs associated with production due to the need for regular freshwater bathing to mitigate the disease. The condition is initiated after colonization of the gills by amoebae which induces the gill epithelium to thicken and results in an excess mucus production due to the multiplication of number of the amoebae attached (Parsons *et al.* 2001) and can be fatal if left untreated (Nowak *et al.*, 2002; Clark *et al.*, 2003; Zilberg and Munday, 2006). The aetiological agent of the disease *N. perurans* (Young *et al.*, 2007), which is present in a close association with hyperplastic gill lesions, is detected using histological and molecular techniques (Dyková *et al.*, 2000; Adams *et al.*, 2004; Young *et al.*, 2007). *N. perurans* has also been reported as the agent for this disease on farmed Chinook salmon *Onchorhynchus tshawytscha* (Walbaum, 1792), rainbow trout, turbot *Scophthalmus maximus* (Linnaeus, 1758), seabass *Dicentrarchus labrax* (Linnaeus, 1758), seabream *Diplodus puntazzo* (Walbaum, 1792), ayu *Plecoglossus altivelis* (Temminck and Schlegel, 1846) and *Seriolella brama* (Günter, 1860) in the marine waters of 8 countries: Tasmania, Ireland, Scotland, United States, New Zealand, Spain (Young *et al.*, 2008b) and Japan (Crosbie *et al.*, 2010) as well as Chile (Bustos *et al.*, 2011). The current treatment in Tasmania is repeated freshwater bathing of salmon during the 14 months of grow-out in seawater (Parsons *et al.*, 2001). This treatment mitigates the disease but does not eliminate the parasite from the farming environment. The presence of AGD is related to water with marine salinities (>33), high temperature and cessation of a normal halocline due to a decrease in rain and superficial freshwater runoff (Adams and Nowak, 2003).

Other pathogens which have been recorded in the Tasmanian salmon industry during marine grow-out phase include *rickettsia*-like organism (RLO) (Corbeil *et al.*, 2003; Corbeil and Crane, 2009), marine *flexibacter* (Handlinger *et al.*, 1997), the non-pathogenic Tasmanian salmon reovirus, (TSRV) (Carlile, 2008; Zainathan, 2012) and a parasitic isopod *Ceratothoa* sp (Copepoda: Isopoda: Cimothoidae) (Nowak *et al.*, 2007). Nevertheless, these pathogens have not caused the serious problems as those produced by *N. perurans* (Crane *et al.*, 2000). In contrast to Chile and many other countries, sea lice have not caused a parasitic disease in salmon in Australia (Nowak *et al.*, 2011).

1.2. *Caligus rogercresseyi* and control strategies

In Chile, *C. rogercresseyi* can live on native fish associated with salmon net pens (Carvajal *et al.*, 1998; Sepúlveda *et al.*, 2004) some of which are its natural hosts. The low host specificity of this species, as with other *Caligus* species (Costello, 1993; Costello, 2006), facilitates its transmission and makes its eradication difficult. In a study of parasitic copepod infections of native fish species living in association with net pens and the parasites on farmed salmon, five species of caligids were identified on wild native fish and only two on farmed salmonids (Carvajal *et al.*, 1998; Sepúlveda *et al.*, 2004). The dominant species on the salmonids was *C. rogercresseyi*, although *Caligus teres* (Wilson, 1905) was also detected but with a prevalence of less than 1%. Fish species like *Eleginops maclovinus* (Cuvier, 1830) and *Odontesthes regia* (Humboldt, 1821) are considered natural reservoirs of C. *rogercresseyi* (misidentified as *Caligus flexispina* in earlier publications) and are responsible for their transmission to farmed fish. These fish inhabit coastal waters where the salmon farms are located (Mann, 1954; Pequeño, 1979; 1981). Nevertheless, the main reservoirs for lice are currently farmed fish (Molinet *et al.*, 2011; González *et al.*, 2012). *In situ* studies using sentinel fish (naïve salmon smolts) experimentally introduced into fallowed sites detected the presence of *Caligus spp.* copepodids and suggested that external infection occurred via neighbouring salmon farms (Rozas and Asencio, 2007).

The first studies on sea lice control on marine salmon farms in Chile focused on the effectiveness of chemical treatments (Sievers *et al.*, 1996a; Roth, 2000; Bravo *et al.*, 2010). However, no control strategy is presently available that completely eliminates the sea louse with all its development stages, and as a consequence re-infection occurs (Boxaspen and Holm,

2001; Costello, 2006; Yatabe *et al.*, 2011). In addition, sea lice have developed reduced sensitivity to the most popular treatment in Chile emamectin benzoate (Bravo *et al.*, 2008a; Bravo *et al.*, 2014), as they have to other compounds elsewhere (Jones *et al.*, 2013). A study on sea louse larvae dispersion and seawater circulation in a bay at the south of Chile determined that the farms were too close together (Molinet *et al.*, 2011). Therefore, to control sea lice re-infections on salmon farms, the application of chemical products has been intensified and an increased number of registered products are being used (Anonymous, 2013). Because the effectiveness of treatments has generally been limited, there have been more studies about the biology of *C. rogercresseyi*, its environmental requirements and farming practices such as fallowing periods after harvesting in order to improve control strategies (González and Carvajal, 1994; Carvajal *et al.* 1998; González *et al.*, 2000, González and Carvajal, 2003).

Like other caligids, *C. rogercresseyi* has a direct life cycle involving eight developmental stages (González and Carvajal, 2003). The first free-living and planktonic phase has three stages, two naupliar stages and one infective copepodid. The second phase is parasitic with four chalimus stages that attach to the host. The third phase comprises pre-adults and mature adults that move freely on the skin of the fish. To design strategic treatment and fallowing regimes, the development of the planktonic and parasitic stages of *C. rogercresseyi* on tank-reared rainbow trout, *O. mykiss*, were examined under natural ambient sea water temperatures (Gonzalez & Costello, unpublished). The hypothesis of the study was that knowing the duration of the life-stages it would be possible the time strategic application of chemical treatments and fallowing to control the parasite on farms. Earlier data obtained from these analyses were used to develop control strategies for Chilean salmon producers (Salmon Chile) in 1999 (Zagmutt-Vergara *et al.*, 2005) and for Sernapesca, the National Fisheries Service (Sernapesca, 2007a; Yatabe *et al.*, 2011; Hamilton-West *et al.*, 2012). These results, together with the industry experience, have led to recommendations for improved control strategies, in particular extending the duration of post-harvest fallowing periods.

The present *Caligus* Monitoring and Control program was implemented in 1999 on salmon farms and began with biological studies to develop control strategies avoiding the use of chemotherapies or at least reducing their impact on fish and the environment (González and Carvajal, 1999). But a control strategy without these chemical treatments was thought to be impossible in this intensive production system. After several years of experience in the salmon aquaculture and of research on this parasite and its control, at present a *Caligus* Monitoring and

Control (Sernapesca, 2007a; 2008; 2012) is compulsory in the whole industry (Sernapesca, 2007b; Yatabe *et al.*, 2011; Sernapesca, 2013). One problem with this control program is that it is based on experience in the northern hemisphere where farmed salmon co-exist with wild populations of the same species. Because the decline of these wild populations that are important fisheries has been associated with the salmon farm activities, social and economic pressures exist to control the release of sea lice from farms (Krkošek *et al.*, 2006; Costello, 2009; Middlemas *et al.*, 2013). In countries like Ireland, Scotland and Canada, the most important sea louse is *Lepeophtheirus salmonis* and less important is the associated species *Caligus elongatus* (Costello, 2006). In those countries farmed salmon are treated firstly, to maintain farmed salmon welfare and secondly, to eliminate sea lice release that are suspected to be one of the factors associated with the decline of wild salmon catches (Crane *et al.*, 2011). In Chile, there are no wild salmonid populations or other fish fisheries to protect from sea lice released from salmon farms, although some wild fish that are not important fisheries can harbour the same species of lice (*E. maclovinus* and *O. regia*). Therefore, the sea louse treatments on salmon farms in Chile only need to focus on the reduction of the parasite for the benefit of their production, but taking into account that these treatments should not be more stressful than the parasite itself.

1.3. Piscirickettsiosis

The infectious disease is caused by *Piscirickettsia salmonis* Fryer, Lannan, Giovannoni and Wood, 1992, an intracellular obligatory bacterium that can be replicated in fish cell cultures within intracellular inclusions such as vacuoles at the cytoplasmic level (Cvitanich *et al.*, 1991; McCarthy *et al.*, 2008). Piscirickettsiosis in fish was first recognized in Chile and remains one of the major diseases affecting salmon aquaculture in that country in the marine, estuarine and occasionally freshwater environments. The disease was first reported in 1989 after causing mortalities in several farms in Chile and was designated as a new disease in the country and around the world (Bravo and Campos, 1989b; a; Alvarado *et al.*, 1990; Fryer *et al.*, 1990; Schäfer *et al.*, 1990; Cvitanich *et al.*, 1991). Epizootics occurred in all salmonid species farmed in Chile, Atlantic salmon *S. salar*, Chinook salmon *O tschawytscha*, masou salmon *Oncorhynchus masou* (Brevoort, 1856) and rainbow trout *O. mykiss* and was then called ‘salmon rickettsial septicaemia’ (SRS) (Cvitanich *et al.*, 1991; Bustos, 2010). Control of the

disease in Chile has not been possible and antibiotics are used to treat it with debatable results. In Norway in the autumn of 1988, mortalities in Atlantic salmon in sea water were attributed to a systemic disease characterized by liver necrosis and was therefore called "necrotizing hepatitis". The liver necrosis comprised of white circular or hemorrhagic foci which were also sometimes scattered in different organs (also in gill capillaries), and were associated with intracellular, intravacuolar bacteria-like inclusions. These inclusions showed affinity for phagocytic host cells and by 1992 a *rickettsia*-like-organism (RLO) was isolated from dead fish. The disease was observed to occur in smolts after exposure to seawater; however they were stocked at high densities and probably underfed as fish were observed feeding on zooplankton (Olsen *et al.*, 1997). Unidentified RLOs have also been reported in freshwater and marine waters of different species in Egypt (1939), Europe, Canada, Wales, Taiwan, Colombia and France (Fryer and Mauel, 1997; Evelyn *et al.*, 1998). Larenas *et al.* (1995) suggested that *P. salmonis* is an immunosuppressive agent that can cause more severe cases when other pathogens (for instance *Renibacterium salmoninarum* Sanders and Fryer, 1980 the causative agent of bacterial kidney disease) are present. Experimental infections in tank-reared coho salmon in freshwater have shown that *P. salmonis* can be transmitted horizontally through the skin, undamaged gills and via vectors as ectoparasites (for example the copepod *Caligus* sp. and isopod *Ceratothoa gaudichaudii* Milne Edwards, 1840) (Smith *et al.*, 1999) and vertically through seminal and ovary fluid and gametes (Larenas *et al.*, 1996). The main routes of the initial infection in natural conditions were proposed to be through injured skin and/or gills (Smith *et al.*, 1999; Yáñez *et al.*, 2014). Due to the septicemic nature of the disease, the pathological signs of the disease in the later stages of infection become similar (Yáñez *et al.*, 2013). Experimental transmission by cohabitation or bath challenge with the pathogen showed lower virulence compared to field infections, and there is a possibility that *P. salmonis* can be transmitted via a marine invertebrate reservoir or vector but this remains to be proved (Birkbeck *et al.*, 2004). As vectors or reservoirs for *P. salmonis* have not been well established in sea water, the main transmission has been considered to be via water. In addition, under field conditions and experimental infection with *Caligus sp.*, *O. kisutch* was shown to be more resistant to the copepod compared to *O. mykiss* and *S. salar*, unless this salmonid species was affected by piscirickettsiosis (González *et al.*, 2000).

1.4. Infectious salmon anaemia (ISA)

Infection salmon anaemia (ISA) is a highly infectious disease of farmed Atlantic salmon which is notifiable to the World Organization for Animal Health (OIE, 2013). The disease caused by the orthomyxovirus *Isavirus* (ISAV) and induces severe anaemia, variable haemorrhages and necrosis in several organs (Cottet *et al.*, 2010). Outbreaks can begin with low daily mortality in few cages (0.5-1%) but can reach very high cumulative mortality (more than 90% in 3 months in severe cases) (OIE, 2013). Clinical ISA was first reported in Norway in 1984 and subsequently appeared in Canada, USA, Faroe Islands and Chile (Mardones *et al.*, 2009) where the disease has not been eradicated yet and Scotland where it was eradicated (OIE 2009). ISAV can infect salmonid species such as brown trout, sea trout (*Salmo trutta*) and rainbow trout, coho salmon and other non-salmonid species without suffering clinical diseases but they can become carriers (Nylund *et al.*, 2007). Also OIE (2013) recognizes the possible creation of virus carriers through vaccination. In addition, the virus has also been isolated from sea water from farm sites with ISAV-positive Atlantic salmon (Kibenge *et al.*, 2004). The main route of virus entry is most probably through gills after shedding of infective material which may be through excretion/secretion by infected salmon (OIE, 2013).

The virus, an enveloped virus, consists of a genome of eight single-stranded RNA segments. Sequence analysis of gene segments (particularly segment 2, 6 and 8) of isolates from Europe and North America has revealed that they can be divided into two major genotypes the European and North American group genotypes. A small highly polymorphic region (HPR) from segment 6 has been suggested to be important for virulence. The full-length gene (HPR0) has been suggested to represent an ancient, non-pathogenic, non-cultivable variant because it has been detected in wild and farmed Atlantic salmon, but not in diseased fish although with no direct correlation (Kibenge *et al.*, 2009; OIE, 2013). All isolates from diseased Atlantic salmon showed deletions in this area. However, other genes are probably important in virulence, because isolates of identical HPRs vary in the eliciting and severity of disease (OIE, 2009).

1.5. Aim of the thesis

The aim of this thesis is to determine the integrated impact of the main parasitic diseases (sea lice and AGD outbreaks) on Australian and Chilean salmon farms. The association of these parasites with the main infectious diseases (ISA and piscirickettsiosis) was described within the salmon farms in Chile, their reservoirs in addition to a comparative analysis of control strategies in Australia and Chile and their effect on outbreaks of major diseases.

To address this question the specific objectives studied were:

1. To identify the presence/absence of Crustacean ectoparasites on farmed Atlantic salmon in Tasmania, Australia, identified them as reservoirs for *N. perurans* and relate it to freshwater treatments (Chapter 2)
2. To determine the environmental reservoirs for *N. perurans* in experimental infections (Chapter 3)
3. To identify the temporal presence of amoebic gill disease (AGD) as *C. rogercresseyi* co-infections on farmed salmon in Chile where a sea lice control strategy was in place (Chapter 4).
4. To characterize the outbreaks of *C. rogercresseyi* infection as co-infection of other important salmon pathogens such as *N. perurans*, *P.salmonis* and ISAV on farmed salmon in Chile (Chapter 5)
5. To determine the presence of ectoparasites on salmon farms in Chile and relate it to antiparasitic treatments (Chapter 5)

CHAPTER 2 GILL ISOPOD *Ceratothoa banksii* AS A POSSIBLE RESERVOIR OF *Neoparamoeba perurans* AND AS A POTENTIAL PROBLEM FOR FARMED ATLANTIC SALMON *Salmo salar* IN AUSTRALIA

2.1. INTRODUCTION

Atlantic salmon *S. salar* were introduced to Tasmania, Australia in the 1800s (Bucklely and Gilligan, 2005). Commercial cultures in netpens began in the 1980s. Although the intensive growth in cages of non-indigenous fish species in the marine environment causes easy transmission of parasites or their vectors in many countries (Kent, 2000), there are few diseases affecting the salmon industry in Australia (Johnson *et al.*, 2004; Nowak *et al.*, 2007). Amoebic gill disease is the main disease affecting the production of Atlantic salmon in this country (Young *et al.*, 2007). The aetiological agent of the disease was identified as the free-living amphizoic marine amoeba *N. perurans* Young *et al.*, 2007. The disease causes hyperplasia of gill epithelium, irritation, excess mucus secretion, hyperplasia and lamellar fusion associated with the amoeba, (Parsons *et al.*, 2001; Adams *et al.*, 2004; Young *et al.*, 2007) and the disease is potentially fatal if left untreated (Munday *et al.*, 2001; Parsons *et al.*, 2001; Zilberg and Munday, 2006; Young *et al.*, 2008a). It was also observed as a response in the interbranchial lymphoid tissue (Norte dos Santos *et al.*, 2014) and in AGD lesions, the absence of chloride cells and an increase in mucous cells (Nowak *et al.*, 2013). AGD has been reported to affect cultured salmonids in different countries (Young *et al.*, 2008b) and is now treated in Tasmania, United States, Scotland and Norway. In Tasmania AGD is treated by bathing fish in oxygenated freshwater for 2-4 hrs (Clark *et al.*, 2003) which reduces the number of lesions on gills (Zilberg and Munday, 2006). Due to constant natural reinfection, freshwater bathing needs to be repeated throughout the year and more frequently during high temperatures. Baths are scheduled commercially by regular 'gill check' assessment of each caged population. Up to 13 baths are required in a typical 15 to 18 month salmon marine grow out period (Kube *et al.*, 2012).

Although crustacean ectoparasites such as sea lice cause significant problems in the mariculture of salmon worldwide, they are not an issue in Tasmania (Johnson *et al.*, 2004; Nowak *et al.*, 2011). Low salinity (<7 psu) affects the development or survival of the sea lice *Caligus roge*rcresseyi killing larvae and causing adults to detach from the host (González and Carvajal, 1999; Zagmutt-Vergara *et al.*, 2005; Bravo *et al.*, 2008b). Therefore, regular

freshwater treatments for AGD may provide additional benefit by reducing crustacean parasitism. Similarly, hydrogen peroxide, a common bath treatment against salmon lice (*Lepeophtheirus salmonis* Krøyer, 1837) is now routinely used to treat AGD on European salmon farms (Adams *et al.*, 2012).

The isopod *Ceratothoa gaudichadii* has caused significant losses on farmed Atlantic salmon in Chile (Inostroza *et al.*, 1993; Sievers *et al.*, 1996b) and another isopod, *Nerocila orbignyi* (Guérin-Méneville, 1829-1832), has been reported to cause problems in cage cultured sea bass *Dicentrarchus labrax* in Greece (Bragoni *et al.*, 1983). However, isopods have not been reported to cause a serious impact on farmed salmon in Tasmania at present although *Ceratothoa* sp has been reported as an occasional finding (Nowak *et al.*, 2007).

The aim of this study was to determine the presence of isopods on both freshwater bathed and unbathed farmed salmon in south-east Tasmania, Australia. The presence of these parasites was also investigated on salmon that had not being bathed for long periods of time. The isopods were collected to screen for the presence of *N. perurans* using PCR and to determine whether these ectoparasites can be reservoirs of the amoeba.

2.2. MATERIAL AND METHODS

2.2.1 Fish sampling

A total of 1355 Atlantic salmon farmed at marine sites in southeast Tasmania were examined for the presence of isopods during commercial gill checks for AGD between 16 December 2009 and 18 September 2012 (Table 2.1). Sampling was performed to cover a range of fish sizes (100g to 6000g) and varying periods since the previous freshwater bath. For examination salmon were randomly seined in the netpen, netted out and anaesthetized in a bath with 17 ppm Aqui-S. They were weighed in groups in the anesthetic bath and fish were counted. Skin, fins, buccal cavity and gills of each fish were inspected for the presence of isopods.

At most samplings, the prevalence (the number of hosts infested with one or more individual isopods divided by the number of hosts examined, expressed as a percentage) and/or mean intensity (the total number of isopods found in a sample divided by the number of hosts infested) of total isopod infection were recorded according to Bush *et al.* (1997). In addition, in December 2009, February and December 2010 different developmental stages of isopod were

collected from gills and skin. The isopods were collected using fine forceps, fixed and stored in 100% ethanol for counting, identification and PCR analysis. Identification of isopods was done following the genus key from Brusca (1981) and the species confirmed by Dr. Niel Bruce (Museum of Tropical Queensland, Queensland Museum and School of Marine and Tropical Biology, James Cook University, Townsville, Australia).

In December 2010, the surface of the second hemibranch was wiped with a sterile cotton-tip swab from 22 of the 83 fish (Table 2.1) with gross AGD lesions according to the method of Young *et al.*, (2008a) and Young *et al.* (2008b). The swabs were stored in RNA preservation solution (RNAlater) for molecular analysis to detect the presence of *N. perurans* as described previously (Young *et al.* 2008a). One or two isopods that were attached to the gills or buccal cavity of each fish were simultaneously fixed in RNAlater and stored at 4°C for molecular analysis.

Baths are scheduled at farms after regular macroscopic "gill checks" salmon indicate a need for treatment. For AGD assessment, subsamples of 40 or more fish were anaesthetised and the extent of visible gill lesions was recorded on a scale of 'clear' (0), 'very light' (1), 'light' (2), 'moderate' (3), 'advanced' (4) and 'heavy' (5) according to gross gill assessment used by Tassal company (southeast Tasmania, Australia) and expressed as an average gill index Taylor *et al.* (2009).

Table 2.1. Monitoring of the occurrence of isopods and AGD on salmon farms in Southeast Tasmania during 2009-2012.

Date	Site	Seawater		Fish		Isopods		AGD					Time since last bathing
		Tem	Salinity	Number	Weight			Number Fish	Scores				
		(°C)	(psu)		(g)	P	I		5 P	3 P	2 P	1 P	(days)
16/12/2009	a	N/A	N/A	343	5500	100	6.0±0.2	N/A	N/A	N/A	N/A	N/A	236
3/02/2010	a	N/A	N/A	104	6000	100	N/A	N/A	N/A	N/A	N/A	N/A	294
15/122010	a	N/A	N/A	83	N/A	100	N/A	N/A	N/A	N/A	N/A	N/A	N/A**
26/3/2012	b	16	32.5	40	2800-3000	2.5	1	N/A	N/A	N/A	N/A	N/A	N/A
				40	2800-3000	2.5	1	N/A	N/A	N/A	N/A	N/A	N/A
26/3/2012	c	16	18	40	100-150	0	0	N/A	N/A	N/A	N/A	N/A	15*
				40	100-150	0	0	N/A	N/A	N/A	N/A	N/A	15*
				40	100-150	0	0	N/A	N/A	N/A	N/A	N/A	15*
				40	100-150	0	0	N/A	N/A	N/A	N/A	N/A	15*
				40	100-150	0	0	N/A	N/A	N/A	N/A	N/A	15*
9/7/2012	d	14.5	32	120	3000	0	0	69	0	3	15	40	97
11/7/2012	d	13.5	32	40	3500	2.5	3	4	0	0	0	10	97
11/7/2012	d	13.5	32	40	N/A	0	0	6	0	0	0	15	97
18/9/2012	d	11.9	32	40	4147	2.5	1	13	0	0	3	30	53
18/9/2012	c	11.5	31	40	1117	0	0	19	0	0	3	45	24
28/11/2012	d	N/A	N/A	40	N/A	0	0	16	0	0	0	40	27
28/11/2012	d	N/A	N/A	225	N/A	0	0	40	0	0	0	0	27

a: Hideaway, b: Meads, c: Killlala, d: Tin Pot. P: prevalence, I: intensity. AGD scores: 5=heavy, 3=moderate, 2=light, 1 very light, N/A: not available. *: Time since transfer from freshwater, **: indicates where the 22 salmon were collected from, for isopod and AGD screening.

2.2.2. Nucleic acid extraction

Samples were removed from RNAlater and processed with the MasterPure™ Complete DNA and RNA extraction kit (Epicentre Biotechnologies). Isopods were analysed for the presence of the *N. perurans* both internally and externally. To detect *N perurans* inside the isopods, firstly each parasite was individually washed and vortexed for 10 s twice with tissue and cell lysis solution from the DNA extraction kit to remove any external amoebae. The washing from each isopod was reserved for further analysis. Then, isopods were dissected to collect a subsample of 10 mg. The aim of determining quantities of the amoeba on the washing solution was to discriminate from false positives due to the presence of *N. perurans* on mucus that normally covered the isopods. Dissected samples, washing solutions and mucus samples on swabs were individually treated for total nucleic acid purification according to the manufacturer´s protocol for tissue and cell samples. In brief, samples were digested with proteinase K at 65°C and proteins collected with the protein precipitation solution (MPC, Epicentre Biotechnologies). The total nucleic acid was precipitated with isopropanol, washed in 70% ethanol and finally eluted in 35-50µl of Tris EDTA buffer (TE buffer).

2.2.3. Quantitative real-time PCR (qPCR) assay for *Neoparamoeba perurans*

The qPCR was performed using the 18S rRNA gene-target primers forward QNperF3: 5′-GTT TAC ATATTA TGA CCC ACT-3′ and reverse QNperR3: 5′-TAA ACC CAA TAGGTC TGC-3′ designed by Bridle et al. (2010). The reaction was performed using the SYBR Green chemistry and an iQ5 Real-time PCR instrument (Bio-Rad, NSW, Australia). Each reaction of 10 µL contained primers 10 µM each, 5 µL of 2x SensiMix plus SYBR fluorescein PCR master mix (Bioline, NSW, Australia) and 2 µL of DNA sample. Samples were tested in triplicate and with no-template control and calibration curve was included on every plate. The copy numbers in the samples were estimated by dividing the copy numbers from the qPCR, by the volume added to the reaction (2 µL), multiplying by the dilution volume for DNA in every sample (35 or 50 µL), multiplying by the extraction efficiency (1.3) reported by Bridle et al. (2010) and multiplying by the dilution factor applied to the sample (1:3, 1:10 or 1:100 diluted in nucleic free water) for the q real- time PCR reaction. The number of amoebae present in the samples was estimated by dividing the copy numbers obtained by 2880, which is the copy number determined in each individual *N. perurans* cell

(Bridle *et al.*, 2010). The quantities were expressed per 10 mg of isopods or per swab in case of gill mucus. The dilutions applied ranged from 1:3 to 1:100 in fish mucus samples due to problems with inhibition during qPCR caused by different amounts of mucus (Table 2.2).

Table 2.2. Sample dilutions to determine copy numbers of 18S RNA segment of *Neoparamoeba perurans* internally and externally from *Ceratothoa banksii* isopods collected from Tasmanian-farmed Atlantic salmon gills on 15 December 2010.

Fish number	Gill swabs	Isopod	
	Sample dilution	Sample dilution	
		External	Internal
1	1:3	1:3	1:10
2	1:3	1:3	1:10
3	1:3	1:3	1:10
4	1:3	1:3	1:10
5	1:3	1:3	1:10
6	1:3	1:3	1:10
7	1:100	1:3	1:10
8	1:100	1:3	1:10
9	1:100	1:3	1:10
10	1:100	1:3	1:10
11	1:100	1:3	1:10
12	1:3	1:1	1:10
13	1:10	1:3	1:10
14	1:100	1:3	1:10
15	1:100	1:3	1:10
16	1:10	1:3	1:10
17	1:10	1:3	1:10
18	1:10	1:3	1:10
19	1:3	1:1	1:10
20	1:3	1:3	1:10
21	1:3	1:3	1:10
22	1:3	1:3	1:10

2.2.4. Statistical analysis

The number of amoebae in the gill swabs was compared to their number inside the isopods using Spearman Correlation (SPSS Statistics package 20 version).

2.3. RESULTS

2.3.1. Isopods on salmon

All Atlantic salmon sampled in December 2009, February and December 2010 were parasitised by isopods (prevalence 100%) when not freshwater bath treated for AGD (Fig. 2.2.). The average intensity in December 2009 was 6.04 $\pm$ 0.17 parasite per fish (Table 2.1). The fish sampled had isopods on gills, buccal cavity, some of them on the skin and on the base of fins. On the skin near the fins few adult isopods were found corresponding to *Nerocila* spp. (Fig. 2.1 a) and on the buccal cavity to *Ceratothoa* spp. and juveniles of both genus (Fig. 2.1 c) according to the description of Brusca (1981). The specimens were identified as *Ceratothoa* sp and *Nerocila orbignyi* (Guérin-Méneville, 1832) by Dr. N. Bruce (pers. comm.). Both species of isopods were present on the gills and buccal cavity, the most abundant being *Ceratothoa* with 114 specimens of adult males and juveniles followed by only 15 juveniles of *N. orbignyi* (Fig. 2.1. b and d). The taxonomic studies at present suggest the identity of the gill isopod as *Ceratothoa banksii* (Leach, 1918) (see Martin *et al*., 2013). Only one reproductive female *Ceratothoa* with eggs and manca were found on the buccal cavity in each of two Atlantic salmon. The AGD level in those fish was not determined (Fig. 2.2.)

Only 1 small isopod (less than 1cm) was found on 80 of the salmon sampled in March 2012 at Meads (prevalence 1.25%, intensity 1), these fish were last bathed in freshwater 35 days before the sampling (Fig. 2.2.). No isopods were detected in 200 fish inspected at Killala 15 days after transfer to the sea site. At Tin Pot in July 2012 where two pens were sampled, only 3 juvenile isopods were observed on gills from one fish form 40 in one cage whereas no isopods were found on any of the 40 fish from the second cage (30 days since bathing). Examination of the gills showed 10% with AGD in first cage and 15% in the second cage both at very light score (Fig. 2.2.).

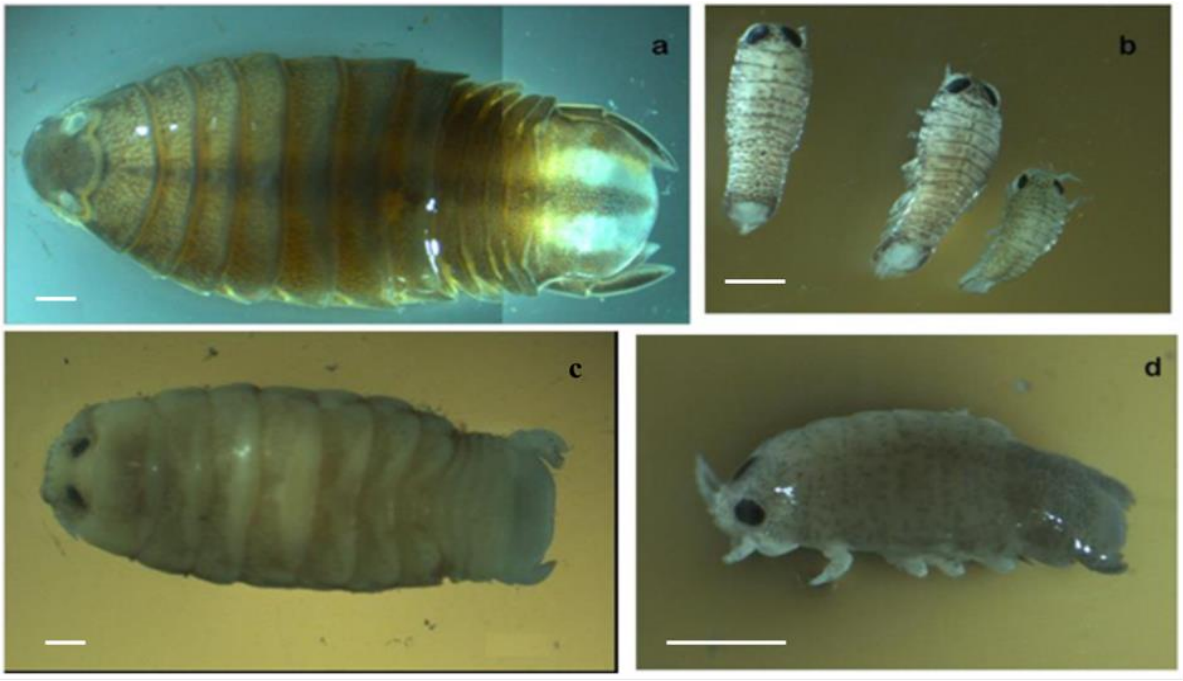


Figure 2.1. Isopods collected from Atlantic salmon farmed in Hideaway Bay, Tasmania, Australia: a) *Nerocila orbignyi* collected from the skin and base of fins of fish, female dorsal view; b) *N. orbignyi*, larval stages (pullus) found on gills; c) *Ceratothoa banksii*, dorsal view of the adult male ; d) *C. banksii*, pullus larva collected from gills together with *N. orbignyi* larvae. (Bars: 1 mm)

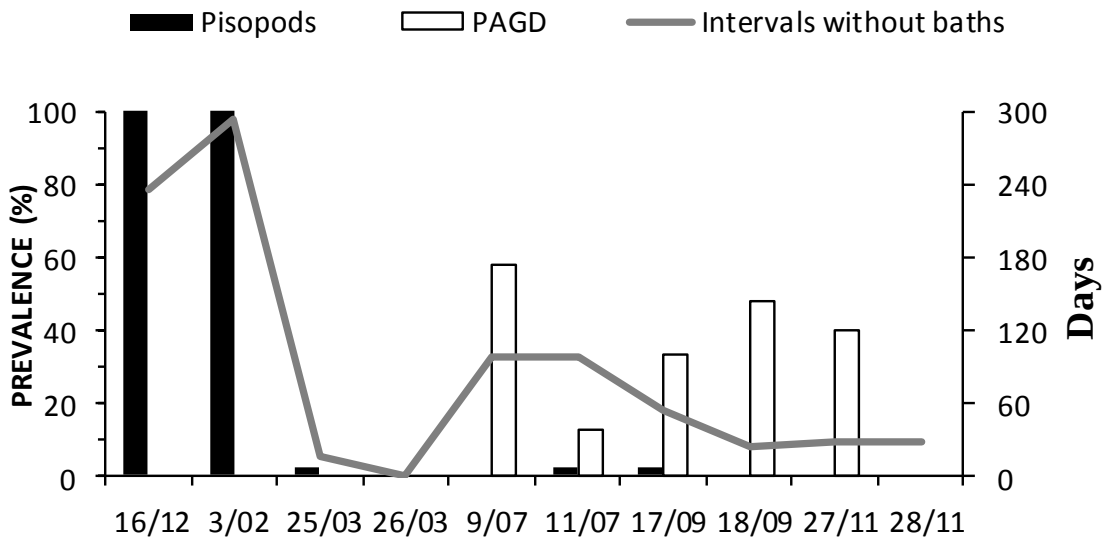


Figure 2.2. Prevalence of isopods (Pisopods) and AGD (PAGD) on farmed Atlantic salmon sampled in Southeast Tasmania during 2009-2012. No data of AGD were recorded during December 2009 and February 2010.

2.3.2. *Neoparamoeba perurans* in gill isopods

Of the 22 fish simultaneously sampled for isopods and evidence of AGD,18 fish (82%) showed presence of *N. perurans* in the gill swabs using qPCR, 9 (41%) of the isopods showed presence of *N. perurans* externally and 2 (9%) of the isopods internally (Table 2.3). Evidence of internal *N. perurans*, was only detected in two isopods in a concentration of 0.3 and 5 cells. When comparing the number of amoebae in the gill swabs to the number of amoebae externally on the isopods from the same gills, it is important to note that *N. perurans* was not found in all the isopods from fish with high concentrations of amoebae in gill mucus and in very low concentrations when it was found. There was no correlation between the quantity of *N. perurans* in the gill swabs and on the isopods (externally r= 0.061 p= 0.393; internally r= 0.284 p= 0.01). In relation to the analysis, all samples needed to dilute out any inhibitory substances for qPCR analysis (Table 2.2).

Table 2.3. Cell equivalents of *Neoparamoeba perurans* (Np) detected on isopod *Ceratothoa banksia* and corresponding gill mucus swabs collected from Tasmanian-farmed Atlantic salmon.

Fish Number	Gill swabs Np	Isopod Np internally	Isopod Np externally
1	278	0	0
2	6	0	0
3	1	0	0
4	0	0	2
5	12	0.3	6
6	2	0	5
7	277	0	1
8	892	0	0
9	7	0	1
10	214	0	5
11	213	0	0
12	0	0	0
13	1	0	0
14	0	0	0
15	908	5	4
16	463	0	0
17	1042	0	0
18	627	0	3
19	6	0	0
20	1	0	0
21	2	0	0
22	0	0	5

2.4. DISCUSSION

This study identified two parasitic isopod species, *Ceraothoa banksii* and *Nerocila orbignyi*, on cage reared Atlantic salmon in southeast Tasmania that have not been freshwater bathed to treat for AGD for more than 7 months. Other species of these cymothoid isopods are considered to be a threat to cage-reared fish (Mladineo, 2003) and may cause considerable damage to the gills and mouth, leaving fish susceptible to secondary infection. In Chile *Ceratothoa gaudichaudii* was observed causing disease in mariculture of coho and Atlantic salmon since 1992 to 1997 (Inostroza *et al.*, 1993; González and Carvajal, 1994; Sievers *et al.*, 1996b; González *et al.*, 1997; Sievers *et al.*, 1997). The isopod was described attached to the inner mouth surface and base of gills feeding on host blood. Sievers *et al.* (1996b) reported that *C. gaudichaudii* caused erosion in the buccal mucosa and destruction of gill lamellae. In addition, it was demonstrated that they caused reduced weight in Atlantic salmon parasitised with 8 isopods or more compared to fish with no parasitic isopods (Sievers *et al.*, 1996b). Mladineo (2003) reported that the haematophagic *Ceratothoa ostreoides* (Risso, 1826) had alternating cyclic periods of blood sucking and blood absorption by the parasite. The absorption periods could bring the required time for fish gills and oral cavity to heal before the next sucking period. *C oestroides* was reported causing progressive growth retardation of 20% and also mortality in cage reared fish (Mladineo, 2003). In the case of *C. gaudichaudii*, isopods have also been noted as a cause of significant losses in Mediterranean farming of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*) (Lanzing and O'Connor, 1975; Horton and Okamura, 2003; Vagianou *et al.*, 2006). In Australia, *C. imbricata* was reported causing a reduction in the weight of wild native fish *Girella tricuspidata* with 2 or more isopods per fish (Lanzing and O'Connor, 1975). Therefore, farmed salmon without freshwater bathing would not only be affected by AGD but also by isopod ectoparasites.

When cages were not freshwater bathed for a long period of time (236 - 294 days), *C. banksii* was the most numerous species on gills and buccal cavity of the sampled fish. These were mainly juveniles, adult males and sporadic reproductive females. Small males or juveniles of *N. orbignyi* were occasionally noted on the gills. The female of *N. orbignyi* and some adult males were located on the skin and base of fins. The prevalence and intensity of *Ceratothoa banksii* found in the present study are similar to the higher levels of *C. gaudichaudii* reported in Chile. Nevertheless, there are no studies on the impact on health or weight variation of

salmon parasitised by the isopods in Tasmania. This is probably due to the fact that isopods are normally very rare and only really seen after winter/spring periods when fish are not bathed for 180 days or more (Taylor *et al.*, 2009).

It is likely that the source of parasitic isopods on Tasmanian salmon is the wild fish that live around the farm sites. Ceratothoids have been described from Carangidae, Mugilidae and Sparidae (Poore, 2002). Records of *Ceratothoa* species include *C. banksii* (*C. imbricata* or *C. trigonocephala* as synonymy) in salmon aquaculture and wild fish in Tasmanian waters as well as *Ceratothoa carinata* (Bianconi, 1869) and *Ceratothoa oxyrrhynchaena* Koelbel, 1878 as new records of buccal-attaching of wild and native fish parasites new to Australia (Martin *et al.*, 2013). *Ceratothoa banksii* are not host specific and have been recorded on cage reared striped trumpeter (*Latris lineata* (Forster, 1801)) held in the Huon Estuary (Andrews *et al.*, 2013).

Freshwater treatment reduces AGD lesions alleviating the physiological perturbations produced by the disease (Zilberg *et al.*, 2001; Clark *et al.*, 2003; Roberts and Powell, 2005). It is likely that the sudden osmotic change experienced when fish are transferred into the freshwater bath is effective in removing mucus and isopod stages from the mouth and skin of salmon. Anecdotally, *C. banksii* have been observed in southeast Tasmanian farmed salmon since at least the mid 1990's and could reach large sizes (50 mm) (B. Nowak pers. comm.). It is possible that these observations were linked as there was no freshwater bathing in the early days of the industry and that the numbers (and maximum size) have largely declined in the intervening years with industry expansion and the operational requirement for frequent freshwater bath rotation to stay ahead of AGD development (R. Taylor pers. comm.). With the potential advent of new treatments and long-term progress of genetic selection for AGD resistance (Kube *et al.*, 2012) which may reduce freshwater bathing events it is possible that parasitic isopods will become a more frequent problem for the industry.

In the Atlantic salmon sampled between December 2009 and February 2010 with long interbath periods that were employed to select potential broodstock from the selective breeding program for AGD resistance (Kube *et al.*, 2012), some sea lice were also found in two cages and reported previously (Nowak *et al.*, 2011). These sea lice (only five specimens, four non reproductive females and one male) were identified as *Caligus longirostris* and were at a prevalence of 1.5 and 1.9% in each cage of salmon. The infrequent presence of very low

numbers of *Caligus* sp. on Atlantic salmon and rainbow trout has been noted commercially in southeast Tasmania over the past 20 years (R. Taylor pers. comm.), but this was the first peer-reviewed report of sea lice on salmon farms in Tasmania after specimens of this species were collected and archived more than 10 years ago from rainbow trout reared in the coast of Tasmania (Nowak *et al.*, 2011). In addition, as in the present study there were no *C. longirostris* ovigerous females, this indicates that farmed salmon in Tasmania were not a suitable host for this parasite to reproduce and multiply as already reported by Nowak *et al.* (2011). The problematic sea lice disease in Chile is caused by *Caligus rogercresseyi* which has a prevalence of 100% (Carvajal *et al.*, 1998), similar to the prevalence observed in this study for *Ceratothoa banksii* in this study.

Haematophagous crustacean ectoparasites are potential vectors of salmonid diseases. Indeed, *C. gaudichaudii* was identified as a potential vector of *Piscirickettsia salmonis* (Evelyn *et al.*, 1998) in Chile as was salmon lice *Lepeophtheirus salmonis* of the AGD aetiological agent *N. perurans* (Nowak *et al.*, 2010). However, this present study indicated that the parasitic isopods do not contain high concentrations of *N. perurans* externally and the amoeba was only found internally in low concentrations in two isopods. Using the quantitative q real-time PCR, five and less than one amoeba was found in each of two isopods where *N. perurans* was detected internally. This indicates that the presence of this amoeba is not common in *C. banksii*. In addition, the content of *N. perurans* in the washing was not always consistent with the presence of this amoeba in the fish gills which could be due to an uneven distribution of *N. perurans* on gills which has been observed in previous histology studies of the disease (Adams and Nowak, 2003; Adams *et al.*, 2004). Swabs, which were taken from a single hemibranch, may misrepresent amoeba numbers on the entire gill by sweeping on areas where either there are few amoebae or by sampling directly from a large lesion. In histopathological studies, AGD-affected gills show discrete and multi-focal epithelial hyperplasia to extensive regions of mucoid patches resulting in lamellar fusion with numerous mucous cells (Adams and Nowak, 2003; Young *et al.*, 2007). Typically *N. perurans* are located on the top of hyperplastic regions or in vesicles (Dyková *et al.*, 2003) and hyperplastic epithelia with numerous mucous cells are rarely colonized by amoebae (Adams and Nowak, 2003). This could explain amoebae presence in the isopod but absence in the swab sample. Thus, a more extensive sampling with the swab on the different gill filaments (or a destructive sample to remove the entire gill basket) would be needed to

accurately reflect amoeba numbers on the entire gill surface. However increasing surface swabbing increases the collection of inhibitory substances for PCR reactions which can influence the results. Considering this later point, the best sampling on fish gills with swabs should focus on the base of filaments where AGD damage occurs; and more restricted to the macroscopic white spots or patches characteristics of AGD.

In relation to isopods as a reservoir of *N. perurans*, the isopod *C. ostreoides* parasite of farmed sea bass, *D. labrax* had been proven to be an obligate blood feeder (Horton and Okamura, 2003). The seasonal ingestion of blood was reported by the color in the parasite gut. But they could have also been ingesting mucus that is translucent and difficult to observe in the gut, particularly in juveniles attached to gills. The ingestion of mucus with *N. perurans* cells which could be partially or totally digested and DNA subsequently degraded by the isopod, could explain the apparent paucity of *N. perurans* within isopods inhabiting the gill surface. This suggests that amoeba within the parasite would be non-viable. Although the presence of adult and juvenile *C. banksii* and juvenile *N. orbygni* on the gills could favor the presence of the amoeba on those ectoparasites, the amount of *N. perurans* associated with the isopods was in general low, despite the sensitivity of the qPCR used to identify and quantify the amoeba. The number of *N. perurans* determined on the washing solution used to clean isopods as a mean to detect presence of the amoebae externally on the isopod, revealed that half of the isopods (41%) were positive for *N. perurans* compared to the 82% of fish samples. It is interesting to note that *N. perurans* was reported in and outside the sea lice *L. salmonis* sampled in Pudget Sound, Washington (Nowak *et al.*, 2010). Therefore, the potential for isopods to act as amoeba vectors is further limited.

The case of the two isopods found with *N. perurans* without presence of the amoeba in the fish mucus could be due to the uneven distribution of amoebae on fish gills, but also could imply that the isopod had moved from one host (with some levels of the amoeba) to another without the pathogen. The life cycle of another member of the same genus, *C. oestroides* and its growth according to their host development stage has been described by Mladineo (2003). The isopod has a direct life cycle and in general requires only one host to mature. Being protandric and hermaphrodite, only one female occurs per host triggered by the settlement of another larva of the parasite. The female produces eggs that develop to pulli. Larval pulli hatch and swim looking for a host. If the host is suitable they grow and then enter the post-larval development. During this part of the development, juvenile isopods go through a male

stage. If there is no female present they develop into female. According to Mladineo (2003) juvenile *C. oestroides* never parasitize adult sea bass and adult isopods cannot migrate from a host to another. Nevertheless, the samplings in March and July 2012 showed that adult Atlantic salmon can be colonized by juvenile isopods. In case to have a similar life cycle for *C. banksii*, only the pullus stage can detach from a fish with AGD and attach to another host thus becoming a potential vector. This life cycle means that only the pullus stages could be involved in *N. perurans* transmission. Nevertheless, the regular freshwater could also affect juvenile isopods preventing their re-attachment to a new fish host.

This is one of the first reports of potential environmental reservoirs of *N. perurans* after the detection of the amoeba in sea water in Tasmania. The study demonstrated that isopods can contain *N. perurans* on the body surface, but the amoebae were in general in low concentrations when the fish host was positive for AGD or free from the pathogen according to quantitative real-time analysis. It is unlikely that *C. banksii* isopods could act as a significant vector of *N. perurans* and transmit the pathogen from one parasitised fish to another host because of the low number of amoebae on isopods as well as juvenile cymothoids. This risk is apparently minimised by freshwater bathing, which has been anecdotally observed to reduce or remove *Ceratothoa* numbers, as indicated by the apparent positive relationship between *Ceratothoa* parasitism and time since last bath. If long-term breeding selection to increase AGD resistance is successful and with the possible advent of alternative (non-freshwater) AGD treatments it is possible that Ceratothoid parasitism will become a problem on Tasmanian salmon farms.

CHAPTER 3 SPATIAL AND TEMPORAL DISTRIBUTION OF N*eoparamoeba perurans* IN A TANK RECIRCULATION SYSTEM DURING EXPERIMENTAL AGD CHALLENGE

3.1. INTRODUCTION

The aetiological agent of AGD was identified as *Neoparamoeba perurans* in 2007 by Young *et al*., (2007) even though the disease has been known for two decades (Munday et al., 2001). *Neoparamoeba* (Page, 1983) are free-living, naked and Lobose amoebae that have been found in many different marine and estuarine habitats. There are few studies on the role of these ubiquitous amoebae in the marine habitats that contrast with the investigations of their role in the soil (Page, 1983). Studies by light and electron microscopy of these amoeabae isolated from the gills of different hosts and localities showed that the morphology of different species was identical except for the size of the trophozoites (Dyková *et al.,* 2000). A characteristic of the amoeba is the presence of one or more intracellular perinuclear bodies (the parasomes) that are eukaryotic endosymbionts but attempts to differentiate members of the genus *Neoparamoeba* using only morphological characteristics have not been successful (Dyková *et al.,* 2000). Using molecular techniques such as *in situ* hybridization, *N. perurans* was identified as the causative agent of AGD in Atlantic salmon (Young *et al.*, 2007). Then, a method based on polymerase chain reaction (PCR) was developed for the diagnosis of the aetiological agent causing the disease (Young *et al.*, 2008a). A PCR-based assay is now essential for the identification of this amoeba because it is specific, highly sensitive and can be used as a routine diagnostic procedure. With this method, the species of the genus *Neoparamoeba N. perurans*, *N. pemaquidensis*, *N. aestuarina* and *N. branquiphila* that are morphologically very similar were finally differentiated (Dyková *et al.*, 2000; 2003).

From an epidemiological perspective, it is essential to investigate the presence of the AGD pathogen (*N. perurans*) in the environment. With the PCR tool it is possible to screen during ecological studies for AGD to detect the presence or absence of this species in the environment. A PCR-base survey on the coast of Washington, United States, was the first preliminary attempt to look for *N. perurans* in the environment around salmon farms (Nowak *et al.*, 2010). However, this survey failed to find the amoeba in biofouling, free-living marine invertebrates and sediments from the marine environment. As the technique used was not sensitive enough to detect very low numbers of the amoebae in the environment, a method

based on highly sensitive real-time PCR analysis combined with sampling method for the water, a total DNA extraction technique and primers specific for *N. perurans* were developed to determine the amoeba distribution in the water around salmon farms positive to AGD in Tasmania (Bridle *et al.*, 2010).

Using the recently developed quantitative real-time PCR-base assay, the purpose of this study was to determine the presence and quantify the concentration of *N. perurans* on different substrates available in a recirculation tank system where experimental infections of Atlantic salmon by the amoeba were occurring.

3.2. MATERIAL AND METHODS

3.2.1. Experimental recirculation systems and inoculation with amoebae

Water samples and biofilm swabs were collected from two recirculation systems where AGD challenges were initiated and maintained on Atlantic salmon for separate studies. The first system consisted of 12 circular 250 L tanks all linked to a common protein skimmer, UV disinfection unit, sump and biofilter with a total volume of 6000 L. The systems were stocked with Atlantic salmon smolts (150 ± 3 g average weight) acclimated to seawater at 25 fish per tank at a density 7.5 kg/m3 for 3 months. The AGD challenge was initiated when cells were harvested from the gills of AGD-affected salmon using the method of Morrison *et al* (2004), then enumerated, equally divided and added to each of the 12 tanks using a watering can to allow for uniform distribution of the amoebae in the water column. Cells were added over three days at concentrations of 100, 217 and 200 amoebae per L for a total dose of 517 amoebae/L. Prior to the addition of the amoebae the recirculation pumps, the protein skimmer and UV unit had been turned off.

Fifteen to 20 min after inoculation, water samples from the bottom of the tank and surface were collected. After two hours of steady state to allow amoebae distribution, the circulation in the tanks was restored except for the protein skimmer and UV system which remained turned off for an additional 24 hours to reduce their effects on the amoebae. The water temperature was approximately 16°C and the salinity 34-35 psu. The system was held in a room illuminated for 24 h and the water quality, including ammonia (0.25-0.5 mg/L), nitrite

(1 mg/L), nitrate (80-160 mg/L), pH (7.4-8.2) and dissolved oxygen (>90% saturation) was monitored daily. Water exchanges occurred as required.

The second system sampled was an individual recirculation tank of 3000 L linked to a protein skimmer, sump and biofilter where AGD was perpetuated by co-habitation of AGD affected Atlantic salmon with healthy and newly added fish. This AGD infection tank has been in operation for more than 4 years and water quality (ammonia, nitrite, nitrate and pH) is measured two times weekly and water exchanges (approximately 30%) occur every fortnight. The biomass in this tank was variable and ranged from 3 -6 kg/m^3.

3.2.2. Collection samples and processing

In the first system on each of the three days of infection and throughout the experiment, water and tank surface samples were collected (Fig. 3.1, Table 3.1). First, the interphase air-water-tank was sampled, by swabbing in duplicate a quarter of the tank circumference in three different tanks each day. The swabs were stored in RNAlater (modified Bridle *et al.* 2010). Then, with each tank in static seawater conditions, 1 L water samples were collected in duplicate at the surface by directly scooping with the plastic container used for their storage, and 1 L at 50 cm deep using a siphon. The siphon was cleaned before and after sampling every day with a 5% bleach solution. Then, after bleaching and between samples in the same day, the siphon was flushed with freshwater at a high pressure to eliminate any contamination, and then rinsed with sea water from the tank before sampling. When the recirculation of water in the system was restored, only the running water of discharge from all the tanks in the sump was sampled. Three samples of 1 L were collected at the running water in the middle of the tank used as a sump, every day for the first seven days and once a week for the following three weeks. Samples in triplicate of the interphase air-water-tank were collected at those dates as well. Finally, 31 days post infection (dpi); the tanks were sampled in steady state for *N. perurans* determination at the surface and at 50 cm depth. During the experiment, moribund and dead fish were removed.

To compare *N. perurans* concentration with the second system where AGD is perpetuated, six samples of 1 L seawater were collected from the 3000 L tank recirculating system.

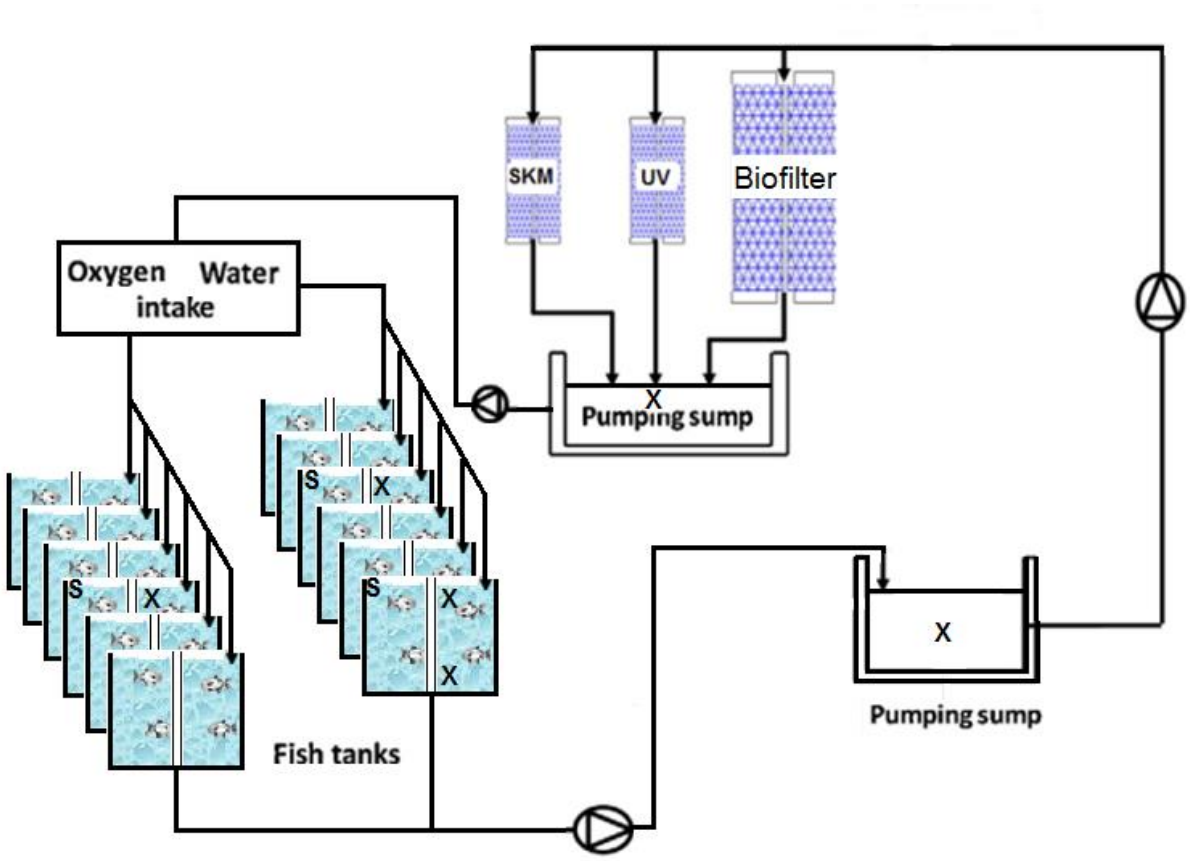


Figure 3.1. Recirculation sea water system with *Salmo salar* where *Neoparamoeba perurans* was experimentally inoculated. The concentration of *N. perurans* was determined in the water column and tank surface on 0, 2, 3 and 31 dpi (days post inoculation). X indicates where the water samples were collected and S, the location of swab samples.

60

Table 3.1: Number of samples collected to determine the density of *Neoparamoeba perurans* in the experimental recirculation system and in the control system where AGD is perpetuated. Number of swab samples taken from the air-water-tank interphase in the tanks, of 1 L sample at the top and 50 cm deep in each tank, of 1 L sample of running water at the sump after the 12 tanks and of 1 L sample of running water at the sump after the skimmer, UV and biofilter.

Time	Tanks				Sump	Comments
	Number	Swabs	Water		Water	
(dpi)		A-w-t	Top	Bottom	Running	
					3	Before adding amoebae
0	3	6	6#	6#	3*	20 min after adding amoebae
					3	
2	3	6	6#	6#	3*	
3	3	6	6#	6#		
4					3	
5					3	
6					3	
7					3	
14					3	
22	3	3			3	
29	3	6			3	
30	3	6			3	
31	3	6	6#	6#	3*	
31	9	18	9#	9#	3*	After the skimmer
99	1		6			AGD infection tank

dpi: days post inoculation,*: after 30 min recirculation, #: after 20 min steady state.

3.2.3. Sample analyses

The swabs taken at the air-water-tank interphase were fixed in RNAlater, maintained at room temperature for 24 hrs and then frozen at -20°C until DNA extraction. All water samples were processed within one to five hours after collection. Each sample was filtered through a 1.2 µm GF/C Whatman glass microfiber filter (GE, Healthcare) using a Venturi vacuum system at low pressure (in a tap water) to collect the amoebae. For DNA extraction the filters were transferred to 15 ml tubes with 2 ml of total nucleic acid (TNA) extraction buffer (0.5% SDS, 10% glycerol, 0.2 M NaCl, RNAse/DNAse free water). The tubes with the filters in the TNA extraction buffer were heated at 55°C for 1 h, mixed by vortex for 10 s every 10-15 min. Then with a transfer pipette the resulting solution of each tube was divided into two tubes, one containing 500 µL and the other tube with the remaining extracted solution to allow enough room for the next adding solutions. Both DNA extracts were first maintained at room temperature for 24 h (mixed as above three times for 10 s every 10-15 min at the end of the period) and then frozen at -20°C. Doing several simultaneously, the samples were thawed at 37°C for 15 min and the extraction proceeded with only 500 µL of each sample. The solution was then treated with a half volume of protein precipitation solution (5M Ammonium acetate, 250 µL co-precipitate pink Bioline) and vortexed for 10 s. Next, they were centrifuged for 10 min at a speed of 10000 x *g* at 10°C, and the supernatant transferred to clean tubes. The DNA was precipitated by adding isopropanol to the samples. It was then washed 2 times in 70% ethanol solution and resuspended in 50 µL of TE buffer. The DNA was quantified with an Invitrogen Qubit fluorometer and Quant-iT dsDNA HS assay kit (Invitrogen, VIC, Australia) and a dilution of 1:3 or 1:5 was performed if necessary. Frozen swabs were thawed at room temperature and centrifuged for 5 min at 3000x *g* and 4°C to pellet cells and swabs which were treated for total nucleic acid purification. Briefly, the RNAlater was eliminated and 500 µL of TNA extraction buffer with 2 µL of proteinase K was added. The samples were digested at 55°C for 30 min, mixed by vortex for 10 s every 10-15 min. Protein was collected by a protein precipitation solution with co-precipitate pink as described above. Then, the TNA was precipitated with isopropanol, washed in ethanol and finally eluted in 50 µL of TE buffer as described above.

3.2.4. Quantitative real-time PCR evaluation for *N. perurans*

The qPCR was performed using the 18S rRNA gene-target primers of 146 bp designed and the protocol of Bridle *et al*. (2010) as described in Chapter 2.

3.2.5. Quantification of 18S rRNA gene copy numbers of *N. perurans* in samples

To determine the 18S rRNA gene copy number of *N. perurans* in the samples, a 642 bp fragment of the gene (position 181 to 822, GenBank accession number EF2169031) was inserted into a pDrive vector (Qiagen, VIC, Australia) and cloned according to Bridle *et al*. (2010). The recombinant plasmid DNA was purified, linearised and their DNA concentration determined using an Invitrogen Qubit fluorometer. The number of copies in the plasmid with the insert was calculated using the software DNA calculator (http://www.endmemo.com/bio/dnacopynum.php) based on the plasmid and insert size of 3512 and 642 bp, respectively. The plasmid-base calibration curve was constructed with 5-fold serial dilutions of plasmid ranging from 3.5174856×10^7 to 45 copies per 1 μL. The samples were tested in duplicate with the standard curve in duplicate. The number of *N. perurans* cells were calculated from the copy numbers resulting from the samples divided by 2 (the template volume), multiplied by 2880 (the number of the 18S rRNA segment copies in *N. perurans*) (Bridle et al. 2010) and by the corresponding dilution factor 3 or 5 of samples.

3.2.6. Statistical analysis

The hypothesis of similar concentrations of amoebae in water samples at different levels of the water column in the steady state water circulation were tested using a Nested analysis of variance (SPSS Inc. 19.0, Chicago, USA). The hypothesis of similar concentration of the amoebae in the running water and swabs collected from the interphase air-water- tank was tested by univariate analysis of variance. The null hypothesis of no differences between population means was tested with an acceptable level of alpha error set at 0.05.

3.3. RESULTS

3.3.1. *Neoparamoeba perurans* distribution in the experimental recirculation system

The density of *N. perurans* was 11 cells/L 3 dpi, what was almost 60 times lower than the amount of amoebae added to the tanks (around 500 cells/L) according to the q real-time PCR analysis. The concentration of *N. perurans* at the top of the tanks with no circulation of water and after 15 min of steady state (Fig. 3.2.) was not significantly different from the concentration of this species at 50 cm ($F_{1,47}$=1.152, p= 0.362). The concentration of *N. perurans* in the water column of tanks at the end of the experiment (31 pdi) was very low or zero in almost all the tanks sampled. On this day, after the water circulation was turned off, the tanks were already disturbed for fish sampling (data reported elsewhere) and Aqui S had been added as a fish anaesthetic and because some fish had already been netted out.

The above data were consistent with the density of this amoeba determined in the running water on days 0, 2 and 3 dpi (1 ± 1, 10 ± 3, 9 ± 2 respectively). During the following days the number of *N. perurans* was very low (1-3 cells/L) until day 15 when an increase was determined reaching 9±3 cells/L. The number of *N. perurans* cells stabilised around 10 to 13 cells/L but with high standard deviations (11 and 7 respectively).

In contrast, the number of amoebae was significantly lower in the samples swabbed from the interphase air-water-tank than in the running water ($F_{1, 59}$: 27.56, p<0.001). The number of *N. perurans* at the interphase water-tank-air was only 0.01 ±0.1 cells/L (on a quarter of tank circumference) on 0 dpi and 0.2±0.2 cells/L on the last day (31 dpi) of the experiment. During the previous days, no amoebae were detected in this interphase.

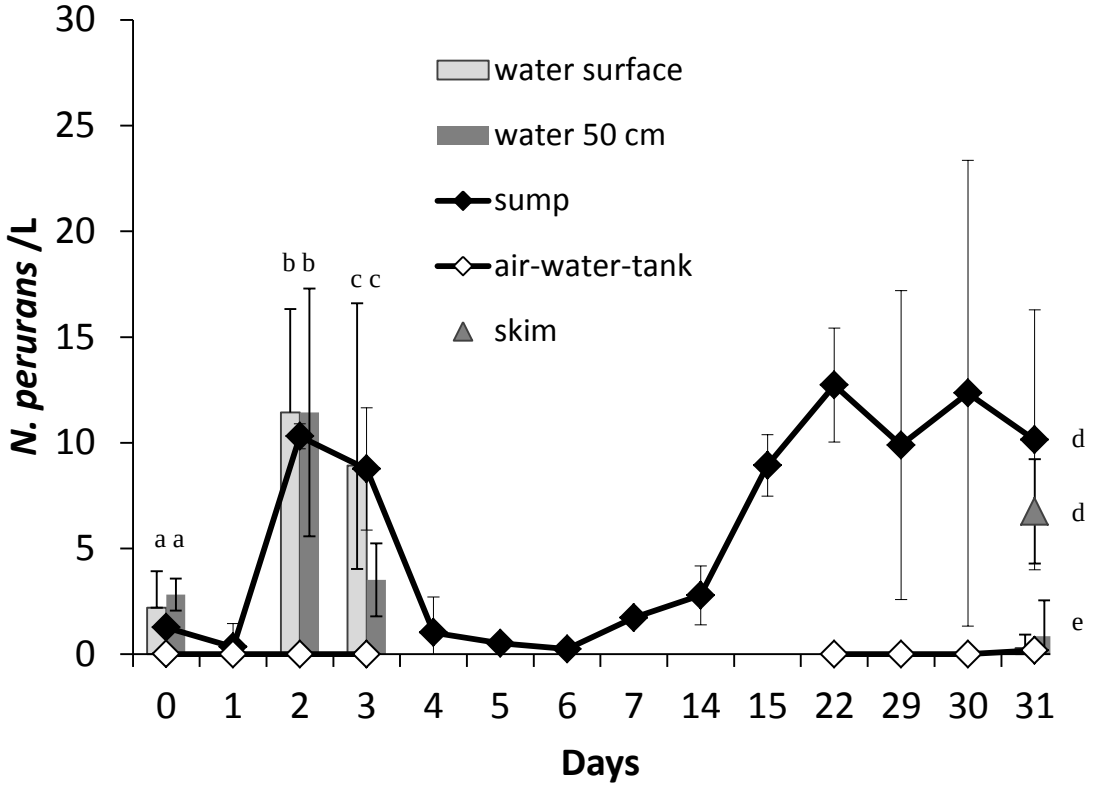


Figure 3.2. Density of *Neoparamoeba perurans* in different parts of the tank recirculation systems stocked with Atlantic salmon, such as at the water surface (light bars), at the water 50 cm deep (dark grey bars), in running water at the sump after the passage through the 12 tanks (black rhombi), at the interphase air-water-tank (white rhombi) and in running water coming from the skimmer after passage through the biofilter and UV unit (grey triangle). (a, b, c, d and e indicate statistical differences of means; same letters means there is no difference between the data, different letters significant differences).

The number of *N. perurans* in the running water after the skimmer or the purifying water system (Fig. 3.2) was not significantly different from the number of amoebae in the running water in the sump or before this water purifying unit ($F_{1,5}$= 5.23, p = 0.08). The density of amoebae was 7 ± 2 after the sea water went through the biofilter, UV sterilization system and the skimmer for protein collection compared to 10 ± 1 cells/L before being treated.

3.3.2. *Neoparamoeba perurans* concentration in the tank where AGD is perpetuated

The number of *N. perurans* in the seawater recirculation system where AGD is perpetuated was 11 ± 0.7 cells/L. Comparing this concentration to the number of cells in the water of the experimental system, using the higher concentrations of cells in the running water, the concentration of *N. perurans* in both systems was not significantly different ($F_{1, 10} = 12.35$, $p<0.968$).

3.4. DISCUSSION

In the present experiment, the concentration of *N. perurans* in the water column in tanks stocked with fish and with a steady state water condition and after 15 – 20 min amoebae were inoculated in the tanks, showed the presence of the amoebae in the water column but no differences between the surface and bottom layer (50 cm). In contrast, the numbers of *N. perurans* in the air-water-tank interphase was zero or almost zero during most the experiment. A previous study determined the distribution of *N. pemaquidensis* (formerly believed to cause AGD) in the sea water column on salmon farms (Douglas-Helders *et al*. 2003). These authors determined the densities of *N. pemaquidensis* using the immune-dot blot technique in 50 and 100 mL samples. Then, Bridle *et al.* (2010) reported this *N. perurans* in sea water around AGD positive farms in Tasmania using qPCR and described it as a free living amoeba. In the present experiment it was shown that the average number of *N. perurans* before the experimental infection grew from zero to a maximum number of 10 ±11 to 13 ± 7 cells/L after 20 days and during the rest of the experiment. Therefore, the maximum concentration of the pathogenic amoeba in the water was much less compared to the concentration found on farmed salmon gills (Gonzalez *et al*., unpublished data). In addition, it is interesting to note that although amoebae were added on the surface of water they do no remain on the upper layer but can distribute throughout the water column in conditions of steady waters and swimming fish. This maximum concentration in the water column is similar to that in the system where AGD is perpetuated by the passage of *N. perurans* from infected to naïve Atlantic salmon by co-habitation but with a lower variability of standard deviation (12 ± 0.8 cells/L) although collected from two recirculation systems with different fish stocks and number of days in culture. *N. perurans* was readily detected by Bridle *et al.* (2010) in the seawater from a commercial Atlantic salmon farm located in the Huon Estuary,

Tasmania, using q real-time PCR. However, these authors reported that the concentration of this amoeba in water samples collected at 0.5 m and 15 m depth was variable and showed no clear association with the gross gill pathology scores assessed on fish. But they did not report the values. In addition in the present experiment, it is noteworthy that fish mortality curve in the 12-tank system reported elsewhere (Dick, 2012) began to be exponential around 23 dpi, implying that the maximum toxic concentration of *N. perurans* for fish was reached. This is probably due to the fact that fish in this system were exposed for the same period of time to *N. perurans* because they were added at the same time at the beginning of the experiment. In the tank where AGD was perpetuated, fish dying from AGD are replaced by the same number of naïve fish, which allowed distribution of mortalities along the time. This system ensures a similar density of amoebae in the water and a constant surface of fish gills for amoebae to settle down, because when fish die due to AGD healthy new ones are added to the system. In the system with the 12 tanks, the stable although oscillating concentration of 10-13 (± 7-11) cells/L 22 dpi in the sea water could indicate the time when exponential fish mortality began; and in case of the experiment, when fish need to be sampled or treated with freshwater bath. The higher variability in the system with 12 tanks could be due to not homogenous distribution of *N. perurans* in the sea water.

In relation to the disinfection units of the recirculation system, their effect on *N. perurans* DNA degradation was unclear. The assessment of the density of amoebae in the sump 31 dpi showed that the number of *N. perurans* was not significantly different than after the skimmer, biofilter and UV where the water had already been cleaned and disinfected. The growing density of this amoeba species after the inoculation with 1-2 cells/L until the stable average maximum average density of 10-12 amoebae/L indicated that the UV and skimmer could not be useful as alternative treatments to eliminate the pathogenic amoeba in these systems. Nevertheless, it could be also possible that amoebae die after the disinfection units but the DNA can still be detected. In that case, the amoebae could be disappearing after a period of time but the average number maintained in the sea water due to the constant multiplication of more amoebae on gills and release to water. Specific trials addressing the utility of disinfection units to treat amoebae in the water would be needed with the proper control systems without the skimmer-UV system.

According to Martin (1985) and Dykova *et al*. (2000), the attachment to substrate is a basic condition for the population of this amoeba to grow. Although the number of *N. perurans* on

fish gills was not determined in the present study, previous studies showed a higher, despite variable, number of *N. perurans* (from 0 to 1042 cells/swab) on farmed Atlantic salmon from Huon estuary chronically affected with AGD (see Chapter 2). The substrate thus, is not an inert substrate such as the tank surface or an animal's shell but most probably fish gills that sieve and concentrate particles where the amoebae can find better prey to feed on (Page, 1983). It has been also reported that higher stocking densities of fish in tanks or cages with an AGD history are related to higher mortalities (Douglas-Helders *et al.*, 2000). There are other records on the environmental reservoirs of the AGD agent *N. perurans*. This amoeba was reported on the sea lice *Lepeophtheirus salmonis* in an AGD positive farm in Washington, North America using conventional PCR that can only determine the presence or absence of the pathogen DNA (Nowak *et al.,* 2010). These authors reported it as a probable reservoir for the pathogen. Nevertheless the previous study in this thesis, on the ectoparasite *Ceratothoa banksii* of salmon aquaculture in Tasmania determined that the isopod was not a significant reservoir of *N. perurans* (see Chapter 1). Although the crustacean can contain the amoeba, this was in general in low concentrations, implying that it is an accidental host for the parasite. These results are in agreement with previous studies using PCR on particles collected from other substrates that failed to identify the pathogen on surfaces such as biofouling, free living marine invertebrates and sediments (Nowak *et al*., 2010).

This is a preliminary study to determine the reservoirs of the economically important pathogen *N. perurans*. Although the results suggested that this amoebae can be found in the water column, *N. perurans* was absent on inert structures such as the tank surface. A concentration of about 10 ± 7 - 13 ± 11 cells/L reached at 22 dpi in this recirculation sea water system were determined. These are lower numbers compared to those found on fish gills that can reach 100 times more. Therefore, morbid farmed fish and those with subclinical levels still remain as the most important reservoir for *N. perurans* pathogenic amoeba. The disinfection units in the system seem not to affect the stability of *N. perurans* DNA although further experiment with the proper control without these disinfection units would be useful.

CHAPTER 4. CHRONIC GILL DISEASE AND CHRONIC SEA LICE INFECTION OF FARMED SALMON IN CHILE

4.1. INTRODUCTION

Amoebic Gill Disease (AGD) was diagnosed on salmon farms in Chile reported with deteriorated status of the production parameters of this intensive aquaculture activity in 2006-2007 (Bustos *et al.*, 2011). Farmed salmon showed low food consumption, high mortalities, poor growth and high *Caligus* infestation. AGD had already been reported in Chile in 1990 in Atlantic salmon affected by piscirickettsiosis (Nowak *et al.*, 2002) and then in 2007 in Atlantic salmon concurrent with new outbreaks of sea lice (Bustos *et al.*, 2001). As the molecular techniques to identify the causative agent were only available since 2007, the presence of *N. perurans* together with the sea lice *C. rogercresseyi* was possible. The outbreak of this sea louse could be occurring as a secondary infection or co-infection of AGD. It is noteworthy that *N. perurans* was reported in association with the sea louse *Lepeophtheirus salmonis* in Atlantic salmon farmed in Pudget Sound, Washington, United States (Nowak et al. 2010).

The aim of the present study was to investigate the cause of gill damage on farmed salmon with sea lice outbreaks and following chemical bath treatments in Chile. The environmental conditions at farm sites that could explain gill diseases were also investigated. The environmental conditions related to the absence or presence of AGD, were reported according to what would provide an insight into their ecology and some oceanographic characteristics in Chile.

4.2. MATERIAL AND METHODS

4.2.1. Farm site

In November 2011, rainbow trout were sampled at Farm 1 (42º 28’ 37’’ Lat. S, 73º 39’ Long W) and Atlantic salmon at Farm 2 (42º 28’ 29” Lat. S, 73º 82’ 49” Long W) in a sea channel between Chiloé big Island and Quinchao Island on the eastern coast of the Chiloé archipelago, Chile (Fig. 4.1 a). The waters between Chiloé Island and continental Chiloé are composed of three water masses, the Sub-Antarctic Water (SAAW) between the surface and

150 m, remnants of Equatorial Subsurface Waters (ESSW) between 150 and 300 m, and Antarctic Intermediate Waters (AAIW) below 300 m (Sievers and Silva, 2008). The first two masses penetrate the Ancud Gulf and channels as far as the bathymetry allows. Narrow and relatively deep channels with many islands limit sea water circulation in this area. The intermediate layer of SAAW enters at the southern end of the large Chiloé Island through the Boca del Guafo into Corcovado gulf. This mass is modified by fresh surface water arriving as far as Seno Reloncaví (off Puerto Montt). The third mass cannot reach all the interior channels because of the topography. As well, the ESSW is blocked at Boca del Guafo by important constriction sills, namely Paso Desertores-Apiao (Sievers and Silva, 2008).

Netpens at Farm 1 were located in a bay with a concave coastal line with 5 brooks opening to the channel and 420 m from several mussel cultures surrounding the farm (Fig. 4.1 c). Farm 2 was 2.7 km far from the previous farm in the same sea channel. Farm 2 was located further from the bay and thus less affected by the brooks discharge. The latter farm was next to a headland where there were neither bays nor rivers present and was surrounded with mussel cultures at one end of the cage rows at a distance of 330 m.

In 2013 Atlantic salmon were sampled at Farm 3 (42°46’ Lat. S, 73°52’ Long W) at the east coast and northern at the Chiloé Archipelago (Fig. 4.1 a). This farm site was further up the Ancud gulf and near Chacao channel and the southern peninsula in an open ocean area. Fish were sampled in March, April and June 2013. The farm was 2.6 km from the mouth of the Hueihue River and 24 km of the channels and islands of the central east of the Chiloé archipelago (Fig. 4.1 b). The farm was also 1.3 km from some mussel cultures. Sea water depth in the site was around -50 m and salmon cages were 30 x 30 m square shaped and 20 m depth (farm data, M.Vet. J. Gatica pers. comm.). Two sets of 12 cages were displayed in two rows (Fig. 4.1 b).

4.2.2. Fish

Only morbid or rejected fish were allowed for collection as samples in 2011 during the removal of dead, sick and rejected fish at Farms 1 and 2. Rainbow trout were farmed at Farm 1, which had an average weight of 2.6 kg. The fish had been raised for 357 days or 3913 ATU (Accumulated Temperature Units for salmonids) in a density of 3.57- 5.12 kg/m^3. Atlantic salmon farmed at Farm 2 had an average weight of 3.8 kg. They were raised for 415

days or 4599 ATU at a stocking density of 8.16 – 12.41 kg/m^3 and were in their second year in the sea. Atlantic salmon were maintained in culture until mid-2012 and then harvested.

In 2011, rainbow trout were sampled on 9 November and Atlantic salmon on 25 November during the routine monitoring for sea lice, which was done weekly in this month due to sea lice outbreak. Three cages were sampled; one in one corner and two haphazard cages in the middle in each sampling. Twenty nine rainbow trout were obtained at Farm 1 (about seven to eight fish per cage) and placed individually in plastic bags for analysis in the processing room of the sea farm. Twenty Atlantic salmon were collected from two haphazardly selected cages and the index cage. The index cage at Farm 2 was the nearest cage to the mussel farms (Fig. 4.1 c). During sea lice counting, fish gills were inspected for gross AGD-gill lesions corresponding to raised, white mucous spots and/or patches on gills according to the description of Adams *et al*. (2004). The second gill arch was swabbed for detection of *N. perurans* in gill mucus according to the method of Young *et al.* (2008a) and a portion of gill was dissected and then, all of them placed in 99% ethanol for molecular analysis.

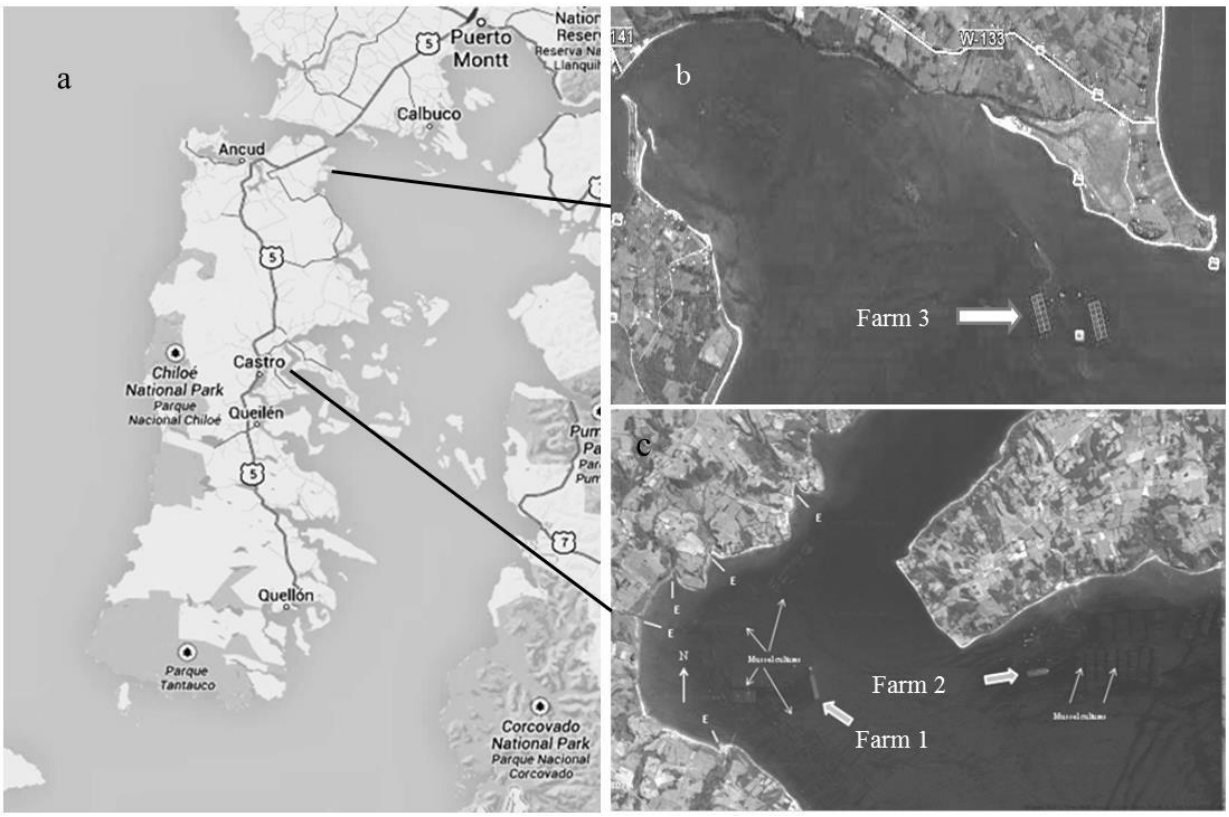


Figure 4.1. Salmon farms sampled on the East coast of Chiloé Archipelago in southern Chile (a). Farm 1 with rainbow trout, Farm 2 with Atlantic salmon (c) and Farm 3 with Atlantic salmon (b). E: brooks. Mussel cultures were present around the netpens (thin arrows).

Fish at Farm 3 were Atlantic salmon and an all-female population. In this farm, only apparently healthy fish were sampled. Fish had an average weight of 2.7 kg in April and 3.8 kg in June and were going through their second winter in the sea. Salmon cages were 20 m depth and square shaped (farm data, M.Vet. J. Gatica pers. comm.) and arranged in two sets of 12 cages in two rows (Fig. 4.1 c). Fish were farmed in a stocking density of 5 to 9 kg/m^3. Due to a rise in mortality in March during the sea lice treatments, fish gill samples were collected on 11 March and fixed in 10% formalin by the farm veterinarian (M Vet. Andrés Santana pers. comm.) and sent to Pathovet Laboratory for histological processing. The slides were examined under light microscopy. AGD was diagnosed in March 2013. On 30 April and 19 June 2013, a random sampling was conducted on fish for diagnosis of AGD during the routine monitoring for sea lice. Twenty four Atlantic salmon were sampled in April and June at Farm 3. Eight fish were collected from each of the three cages in the southern row netpens

(a cage in one corner, the index cage in the other corner and a cage in the middle). In June, eight fish were collected from each of the three cages (two cages in the corners and one in a middle cage). Fish were netted out and transferred to a bath containing an overdose of benzocaine (BZ 20, Centrovet Chile). Each fish was individually placed in a labelled bag. Inside the bag, a section of the second gill arch of the left gill was excised and immediately fixed in 40 mL of Davidson's formaldehyde solution. Then, a portion of 10-5 mg of gills was dissected and placed in RNAlater. For AGD analysis, samples were transported to Universidad de Santiago and a backup sample to Pathovet Laboratory (Puerto Montt). Samples for molecular analysis were frozen at – 20 °C except for the swabs used to collect fish gill mucus that were maintained at 4ºC.

4.2.3. Histopathology

Five gill samples of the rainbow trout at Farm 1 and five of the Atlantic salmon at Farm 2 that were fixed in 99% ethanol approx. 1 hr after collection, were sent for histology processing to ADL Diagnostic Laboratory (Puerto Montt, Chile). The samples were cut into 5 μm sections and stained with routine hematoxyline-eosine (H&E) for histological analysis. Gills from the 2013 samples were fixed in the Davidson's solution for only 24-48 hrs and then transferred to 10% formaldehyde for 24 hrs or more. In the laboratory, a portion of gills was dissected and transferred to 70% ethanol for routine histological processing. Gill sections were dehydrated, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin-eosin. The sections were examined under light microscope for hyperplastic lesions, numbers of mucous cells, fibrosis, cartilage hyperplasia or other histopathology apart from AGD lesions. Gills from the 2013 samples were also screened for gill filaments with lamellar hyperplasia, other cell inclusions or abnormalities.

The proportion of gill filaments with lamellar lesions due to AGD (Adams *et al*, 2004) were compared with the proportion of other lesions or normal filaments on three filaments in a Photo-microscope Nikon and Image Pro Plus analyser at Universidad Católica de Chile (Santiago). Filaments were included in the analysis only if the central venous sinus was visible (in most of its lengths) and lamellae on both sides were the same length. Lamellar damage was classified according to Adams and Nowak (2003) description of progressive stages for AGD pathology with modifications and adapted to other pathologies (Table 4.1).

Table 4.1. Histopathological classification of gill lesions.

Score	Description
0	Normal structure of filament, lamellae, presence of few mucous cells and thin central venous sinus.
0.5	Increase in the number of mucous cells, oedema.
1	Increase in the number of mucous cells, thickening of the basal filamental region (hypertrophia).
1.5	Increase in the number of mucous cells, thickening of the basal filamental region (hypertrophia). Oedema.
2	Increase in the number of mucous cells, thickening of the basal filamental region, clubbing of distal regions of secondary lamellae.
2.5	Increase in the number of mucous cells, clubbing of distal regions of secondary lamellae.
3	Hypertrophy and hyperplasia of epithelial cells and/or desquamation of surface epithelial cells. New reduction in numbers of chloride cells. Begin of hyperplastic epithelium between lamellae.
4	Hyperplasia progression. Fusion of secondary lamellae simultaneously with oedema of the primary filamental epithelium. The basal epithelia of the primary filaments remove to the distal regions of the secondary lamellae.
5	As lesions developed in length along the primary filament, the leading edge of the lesions was preceded by oedema and leucocyte infiltration. Mature lesion with presence of hyperplastic epithelium between lamellae and vesicles with/out amoebae.
6	Complete hyperplasia with vesicles with/out amoebae.
7	Stratification of epithelial tissue located on the surface of a spongiotic, mature lesion and on the top, mucous cells. Larger lesions consisted mainly of undifferentiated epithelial cells and variable inclusion of mucous cells.

4.2.4. Environmental data and water sampling

Water quality parameters other than phytoplankton abundance and temperature were assessed only once. Water samples were collected by a diver in 0.5-L containers at 0-1 and 10 m at 3 points around the cages at both farm sites on 11 November 2011. The samples were analysed within 1 to 2 hours after collection with a Sera Aqua-test Kit (GmbH, Germany) to determine the seawater concentrations of ammonia, nitrates, nitrites, phosphates, copper, alkalinity kH and calcium. Temperature was regularly measured at three sites by the farm staff using a Hanna probe. These data and phytoplankton monitoring data from water samples collected at 0-1 m and 10 m between December 2010 and November 2011 were provided by the farm. Using 1-L bottles, water samples were collected from three different points at each farm at 0-1 and 10 m depths. The samples were fixed with Lugol and analysed by the microbiology and chemical laboratory of the farm company (Ancud, Chile) using an inverted microscope (HI-Tech XDS-2, VHTM Vision Hi-Tech Machines, Madrid). At Farm 3 no phytoplankton monitoring data were available.

Another 12 seawater samples were taken on 12 November 2011 to identify *N. perurans.* The samples were collected in 1-L plastic containers from four corners of each site at depth of 0-1, 5 and 20 m. The samples were filtered through 0.22 μm Millipore with a vacuum pump and each filter was stored at 4°C in Tissue and Cell Lysis Solution (Master Pure complete DNA and RNA purification kit, Epicentre Biotechnologies, Madison, WI, USA).

A CTDO profile of the water temperature, oxygen and salinity in the water column were recorded by Plancton Andino Laboratory (Castro, Chile) to identify any variations in the water column (using a SeaBird Electronics Inc., Model 19 Plus, Bellevue, WA, USA) at three points near Farm 1 during high tide on the evening of 18 November 2011 and ebb tide on the morning of 19 November 2011.

4.2.5. Molecular analysis

4.2.5.1. Total nucleic acid extraction

Total nucleic acid was extracted from sea lice, filters and Tissue and Cell Lysis Solution obtained from seawater samples as well as swabs and a portion of 5-10 mg gill samples, using a MasterPure^{TM} Complete DNA and RNA Extraction Kit (Epicentre) according to the

manufacturer's protocol as described in Chapter 2. The DNA and RNA pellets were resuspended in 40 to 100 µL of nuclease-free water.

In 2013, DNA were extracted from samples at Universidad de Santiago as described and subsampled as backup in Pathovet and ADL Laboratories (Puerto Montt, Chile) for the identification of *N. perurans*.

4.2.5.2. Real-time PCR for *N. perurans*

The 18S rRNA gene segment of *N perurans* was amplified by real-time PCR using the primers QNperF3: 5′-GTT TAC ATATTA TGA CCC ACT-3′ and QNperR3: 5′-TAA ACC CAA TAGGTC TGC-3′ developed by Bridle *et al.* (2010) and the primers (Np18SF1) 5'-CTT ACT AGA CTT TCA CTA TTA CAC-3' (Np18SR2) 5'-TCT AAG CAG AAC GAA CTT TC-3'developed by Rozas *et al.* (2011). In 2011 samples, the gene segment was amplified in volume reactions of 20 µL using SensiMix SYBR chemistry in a Stratagene Mx3000P Agilent Technologies Multiplex Quantitative PCR Systems. The reaction included 10 µl of 2x Brilliant III SYBR Green, 1 µL of 10µM QNperF3 primers, 1 µL of 10µM QNperR3 primer, 0.3µL of Rox (1/500 dilution), 2 µL of DNA sample and DEPC treated water to adjust a final reaction volume of 20 µl. Master Mix reactions were prepared in a special cabinet where no nucleic acid had been handled. DNA samples were compared to negative controls without template and positive controls containing linearised plasmid DNA, housing the 18S rRNA gene segment of *N. perurans*. Plasmid-based positive controls were included in every reaction plate and all the samples were tested in duplicate. As an additional confirmation, the real-time PCR products were electrophoresed through 1.5% agarose in TAE (Tris-acetate-EDTA) buffer stained with ethidium bromide. The temperature profile for the real-time PCR reaction was 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min. At the end of the 40 cycles a melt curve analysis was performed (95°C for 1 min, 55°C for 30 s and 95°C for 30 s) to test the reaction specificity**.**

Samples collected in 2013 were analysed using a master mix with a total volume of 10 µL containing 0.3 µM Taqman probes and 0.9 µM of primers Np18S F1 and Np 18S R2. DNA templates were compared in a Light Cycler S480 (Roche Diagnostics, Germany) to positive samples containing a synthetic amplicon Roche and negative samples corresponding to only the extraction solution of samples. The temperature profile for the real-time PCR reaction in

2013 was 1 cycle at 94°C for 3 min, followed by 35 cycle of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, and 72°C for 10 min.

4.2.6. Statistical analysis

The prevalence of AGD including *N. perurans* presence was determined according to Bush et al. (1997).

Gill histological data were tested for homogeneity of variance using the Levene's test and plot of residuals in the analysis of variances. Then, they were analysed to determine if there was any significant difference in the damage of gill filaments of fish and chemical bath treatments for sea lice in April compared to only bath treatments in June (fixed, orthogonal) of fish in three pens in the farm (nested and random factor) using a non-parametric Mann-Whitney U test for un-related samples (SPSS Inc. 21.0, Chicago, USA). The 10 ranking scores of gill damages were used first for rejection of the null hypothesis of no differences in the proportion of damaged gills between months and between cages. Then, the samples were pooled in the ranks "mucus" gathering ranks 0, 0.5, 1.5 and 2.5 and "hyperplasia" gathering ranks 1, 2, 3, 4, 5 6. A Mann-Whitney test was performed to these new data with value of significance of 0.05 for the rejection of the null hypothesis.

The kH and Ca^{2+} concentration was compared in the water samples of both farm sites using a nested analysis of variance (SPSS Inc. 19.0, Chicago, USA). Results were considered significant if $p<0.05$.

4.3. RESULTS

4.3.1. Gross pathology signs

Rainbow trout at Farm 1 showed gross deformities of the gills with haemorrhaging filaments, excess mucus and sometimes shortened operculum (Fig. 4.2 a). At Farm 2, Atlantic salmon gills appeared normal with no signs of mucous spots or patches (data not shown). Nevertheless, excess mucus production on gills among Atlantic salmon in March 2012 was observed by the farm staff, which could not be sampled for this study (Fig. 4.2 b). In relation to Farm 3, no sign of raised, white mucous spots or patches typical of AGD were observed in the inspection of Atlantic salmon gills in April and in June 2013.

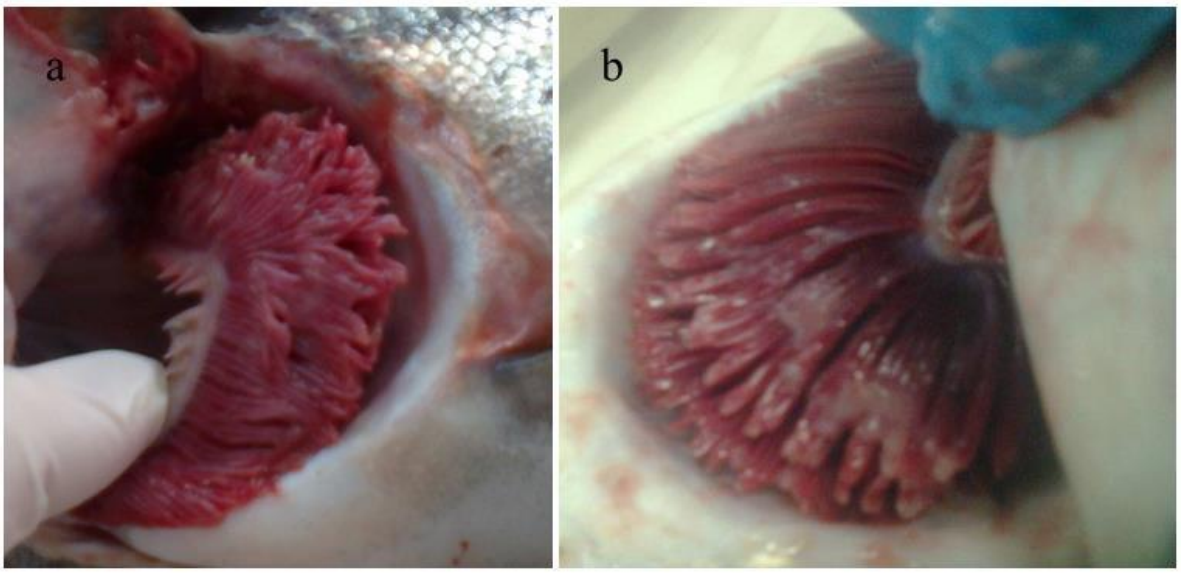


Figure 4.2. a) Gills of rainbow trout in November 2011 at Farm 1 and b) Gills of Atlantic salmon at Farm 2 in March 2012.

4.3.2. Histological analysis

Histological sections from fish sampled in 2011 showed several areas with necrotic lamellae and hyperplasia of lamellar epithelium and several hypertrophic mucous cells. Several filaments showed abundant mucous cells along the lamellae containing eosinophilic and granular substances (Fig. 4.3. a). Nevertheless, due to the poor fixation the percentage of gill filaments affected by the lesions and the number of mucous cells could not be quantified.

Histological sections provided by Pathovet from the gills sampled and analysed on March 2013 showed 33% (5/15) AGD prevalence (Fig. 4.3. b) and the samples from April, 29% (7/24) of prevalence (Fig. 4.3. c). All fish sampled had some degree of hyperplasia of lamellar epithelium but only those showing amoebae were considered AGD positive. The quantification of lesions in April 2013 showed significantly higher proportions of the gill filaments in ranking 1, 2, 3, 4, and 6 with median values 2 (Fig. 4.4. a). The fish with these conditions that were pooled together (Fig. 4.4. b) and named "hyperplasia" showed many lamellae with a large number of mucous cells and sometimes oedema. The quantification of lesions in June showed a significantly higher proportion of filaments in ranking 0, 0.5 and 2.5 with median values in 0.5. These were the samples that were pooled (Fig 4.4. b) and named "mucus". In June there were no vesicles, amoebae and hyperplasia of lamellar epithelium present in any samples. Mucous cells, lamellae clubbing and some oedema were present (Fig. 4.3. d). Hypertrophy of epithelial cells was found along the lamellae (Fig. 4.3. d).

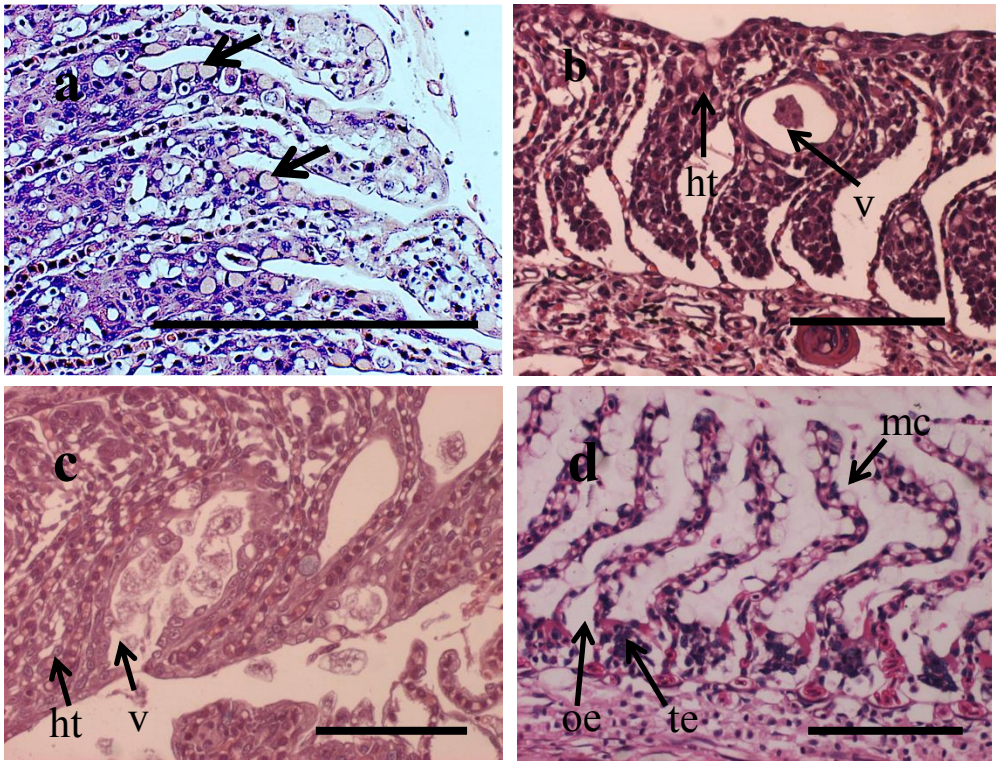


Figure 4.3. a) Rainbow trout with hypertrophic lamellae at Farm 1 in November 2011; several mucous cells with eosinophilic and granular content (arrows) were present; b) Atlantic salmon with AGD lesions at Farm 3 in March 2013 due to epithelial hyperplasia, lamella fusions and vesicles with amoebae; c) Atlantic salmon with AGD lesions in April 2013: lamellar fusions due to hyperplasia (ht), interlamellar vesicles with amoebae (v); d) Atlantic salmon with hyperplasia of mucous cells in June 2013 increase in the number of mucous cells (mc), clubbing of distal part of lamellae, thickening of the basal epithelium of filament (te) and mild oedema (oe). (Bar= 100 μm).

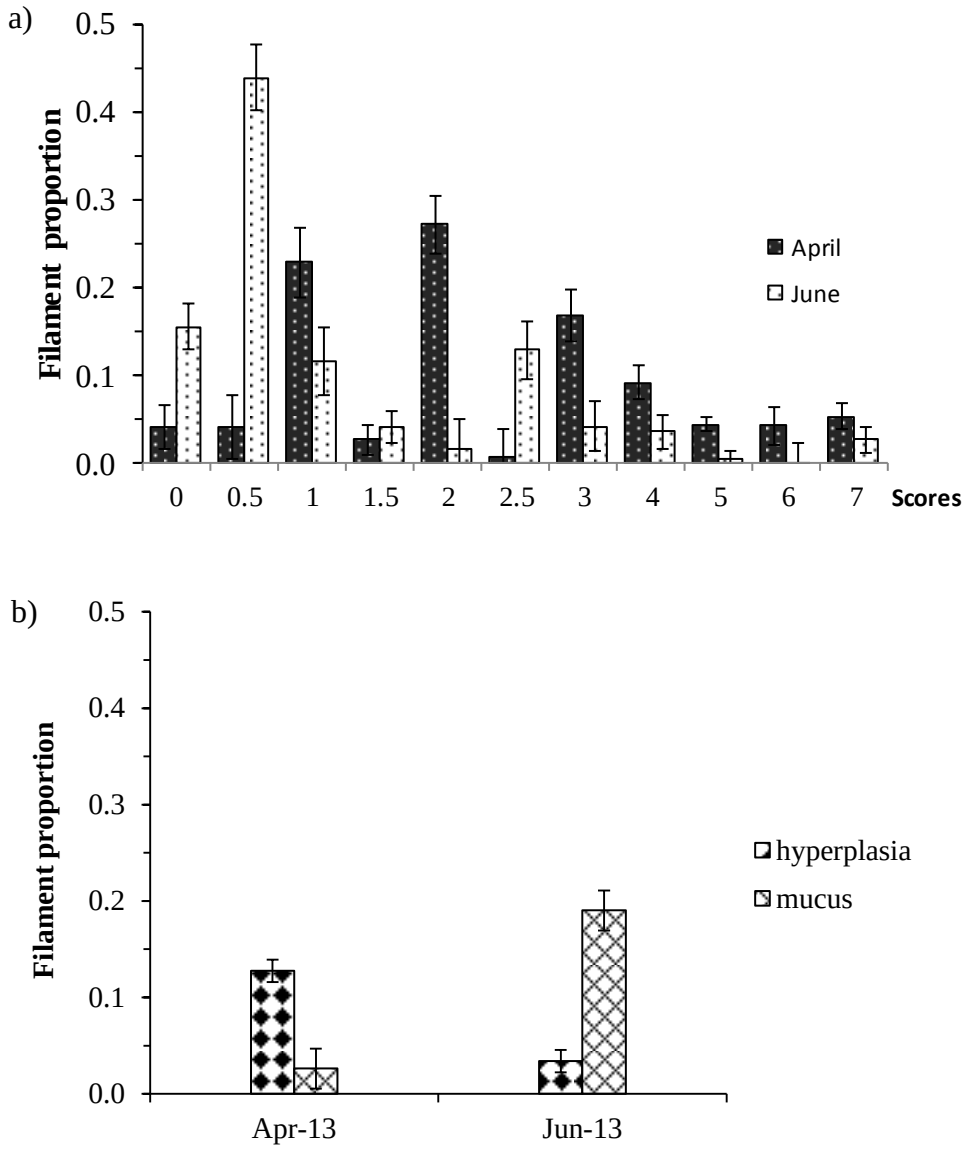


Figure 4.4. a) Distribution of gill pathology on fish sampled in April and June according to the 11 histopathological scores and b) combining the predominant scores (hyperplasia: 1, 2, 3, 4 and 6; mucus: 0, 0.5 and 2.5) in April and June.

4.3.3. Water quality

No differences were observed between Farms 1 and 2 in the concentrations of ammonia, nitrates and phosphates (Table 4.2). The pH levels of 8.2 for Farm 1 and 8.3 for Farm 2 were similar and within the normal range for seawater. Ammonia was lower than the 0.5 mg/L tolerance limit for rainbow trout (Klontz *et al.*, 1985; Timmons *et al.*, 2002). Carbonate hardness (kH) was lower at Farm 1 at 1 m ($F_{1, 11:}$ 5, p= 0.049), but levels at both farms were below the normal level of 8-8.5 kH for seawater, although still within the neutral value of 6-8 (Sera kit manual, GmbH, Germany). The nitrite and Cu concentrations were nil or below the detectable levels of the Sera kit. In relation to Ca^{2+} concentration, the other hardness variable, the nested ANOVA showed no differences between different depths (1 and 10 m) at the same site ($F_{1, 11}$: 0.67, p=0.438), but significant differences existed between sites. Farm 2 had a higher Ca^{2+} concentration than Farm 1 ($F_{1, 11}$: 6.00, p=0.04). However, both concentrations were within the normal range according to the Sera kit manual.

Table 4.2. Concentration of carbonates or alkalinity (kH), ammonium/ammonia, nitrites, nitrates, phosphates, copper and calcium (or hardness) at three points at 0-1 m and 10 m depth at Farm 1 (a) and Farm 2 (b). E: east, W: west, N: north and S: south.

a

Farm 1	1 m			10 m		
	E	W	N	E	N	W
Carbonate hardness kH	6	6	6	7	7	7
NH_4^+/NH_3 (mg/L)	0.25	0.25	0.25	0.25	0.25	0.25
NO_2^- (mg/L)	0	0	0	0	0	0
NO_3^- (mg/L)	0	0	0	0	0	0
PO_4^- (mg/L)	0.1	0.1	0.1	0.1	0.1	0.1
Cu^{2+} (mg/L)	0	0	0	0	0	0
Ca^{2+} (mg/L)	380	380	380	380	380	380

b

Farm 2	1 m			10 m		
	E	NW	SE	E	NW	SE
Carbonate hardness kH	7	7	7	7	7	7
NH_4^+/NH_3 (mg/L)	0.25	0.25	0.25	0.25	0.25	0.25
NO_2^- (mg/L)	0	0	0	0	0	0
NO_3^- (mg/L)	0	0	0	0	0	0
PO_4^-(mg/L)	0.1	0.1	0.1	0.1	0.1	0.1
Cu^{2+} (mg/L)	0	0	0	0	0	0
Ca^{2+} (mg/L)	420	400	420	440	400	420

At Farms 1 and 2 phytoplankton was monitored by the farms when some increase was detected, focusing on diatoms and dinoflagellates. Phytoplankton density (Fig. 4.5) was in general below the warning concentrations for fish safety according to farm laboratory protocol, except for a few dates when it was similar to or above the safe limits. No dinoflagellates were reported. Diatoms, in particular *Chaetoceros* spp. exceeded the maximum level according to the protocol of 300 cells mL^{-1} in January 2011 at Farms 1 and 2 and at only Farm 1 in November 2011, when the samplings for this study were carried out. The concentration of phytoplankton was always higher in the samples at 1 m than at 10 m in the water column at Farm 1. However at Farm 2; the concentration of phytoplankton was higher at 10 m than at 1 m in two dates of three. This implies more mixing in the water

column in this site. The Secchi disc of water depth visibility that is useful to indicate phytoplankton density in the field was higher at Farm 1 than at Farm 2 in January, September and the beginning of October 2011. In relation to Farm 3, no records of phytoplankton were available.

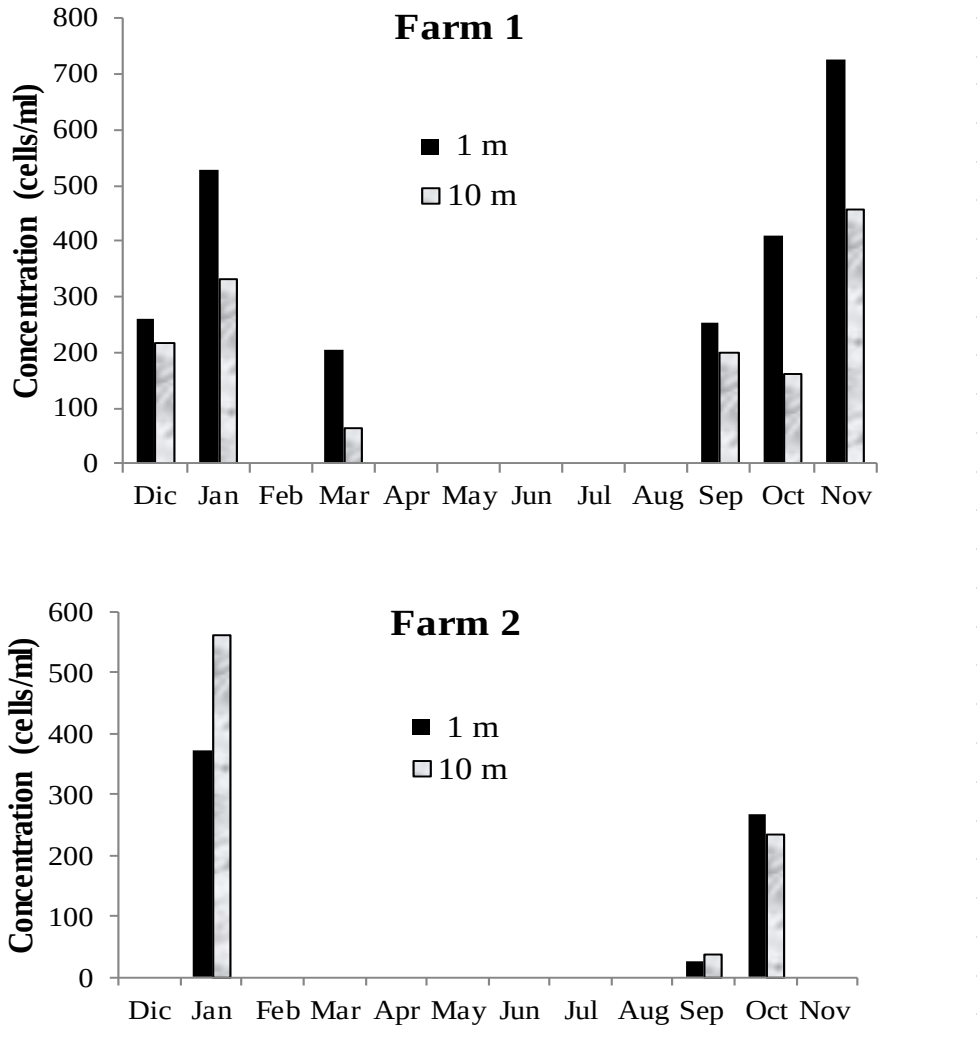


Figure 4.5. Concentration of diatoms (cells/ml) at 1 m and 10 m recorded at Farms 1 and 2.

The long-term temperature data recorded at Farm 1 were similar to the data recorded in the two days with the CTDO (Fig.4.6 and 4.7). In contrast, the recordings for the oxygen concentration were lower in the farm data (corresponding to water inside the cages) compared to the data logged by the CTDO on two days in November 2011 (outside the

cages). The mean oxygen concentration data collected by the farm was 8 ± 1 mg/L at Farm 1 as well as at Farm 2 with similar values at 1 m and 10 m depth. The increase in almost 2 mg/L in the first 5 m recorded by the CTDO profile outside the cages was not present in the water samples regularly taken at 1 m and 10 m inside the cages at both sites. The CTDO downcasts were done out of cages due to possible problems with the nets and ropes. In the long-term data at Farm 1 the oxygen was over 9 mg/L in January at 1 and 10 m and over 8 in March, but was below 8 in September, increasing in October and November again over 8 mg/L. At Farm 2, the oxygen reached 6-7 mg/L in January at 1 and 10 m respectively, but then increased to over 8 in September and October. The daily records of temperature at Farm 3 showed variability from 15° to 12°C at 5 m from January to March (the summer months) (Fig. 4.7). From April to June (the autumn months), the temperature and variability steadily decreased from 12° to 11°C. In the winter months, from July to August the temperatures stabilized at around 10°C. The Secchi disc visibility was higher (over 6 m) in January and February, decreasing during the three autumn months from 5.4 to 4.3 m. In winter months the turbidity stabilized around 4.6-4.7 m. These values matched to the high levels of oxygen in January and February (8.5 and 6.9 mg/L respectively) that then dramatically decreased in autumn to 5.4-5.0 mg/L. In June the oxygen increased again to 5.8 with higher levels in July with 6.7 mg/L.

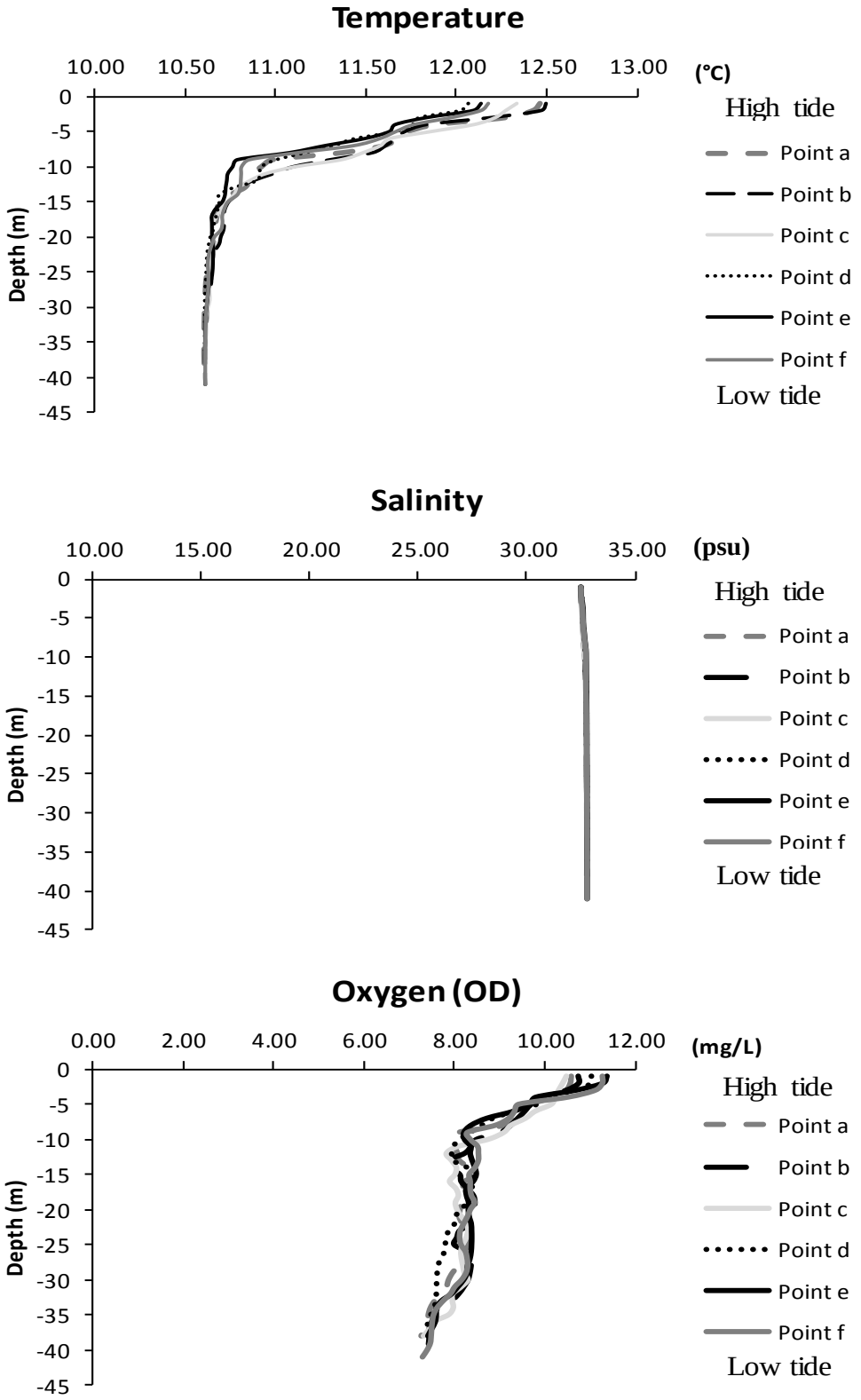


Figure 4.6. Oceanographic parameters measured with a CTDO, a) temperature profile, b) salinity profile and c) oxygen concentration.

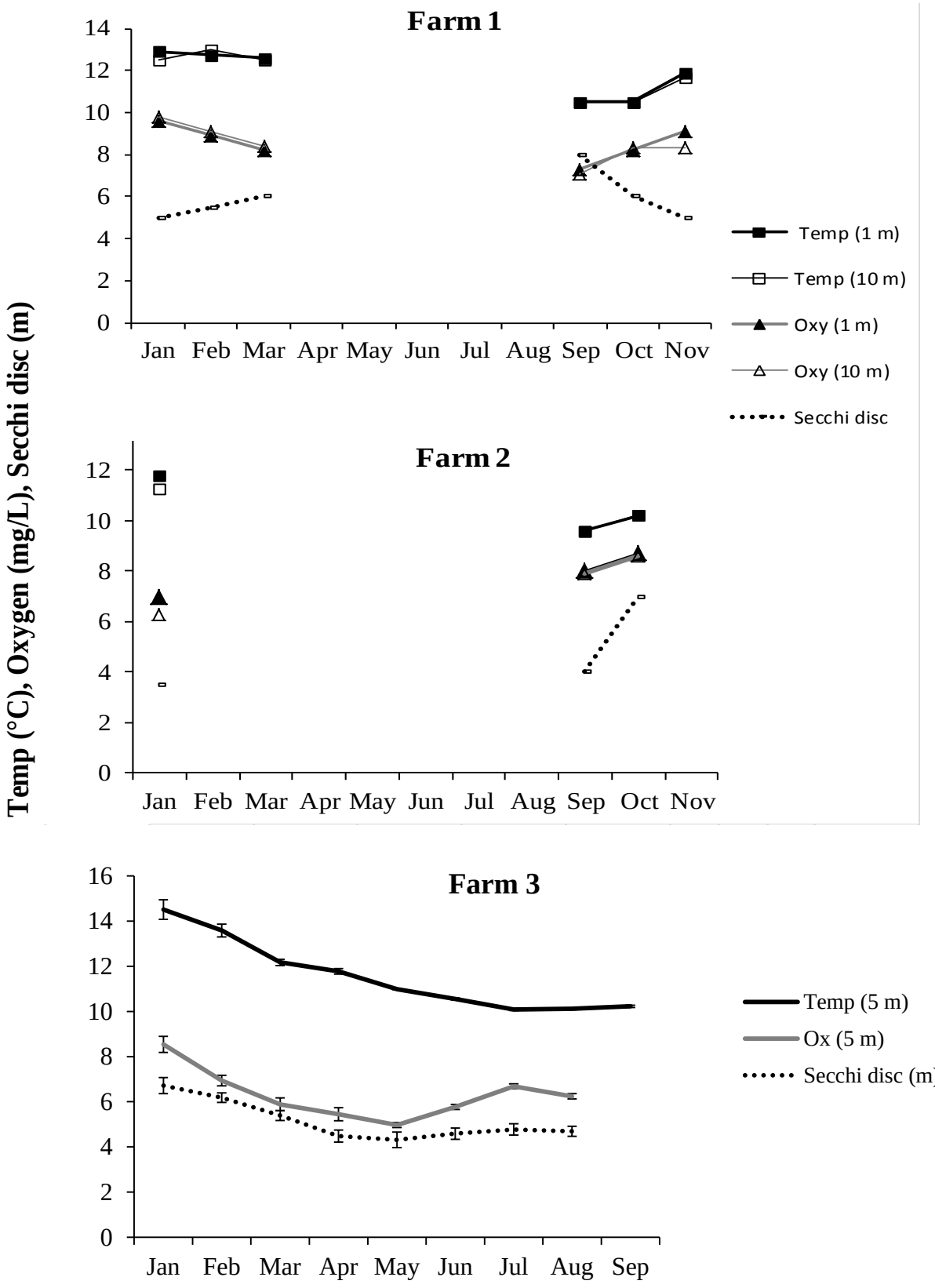


Figure 4.7. Temperature, oxygen concentration (mg/L) and Secchi disc visibility at 1 m and 10 m at Farms 1 and 2, and 5 m at Farm 3.

4.3.4. Molecular analysis of samples

In 2011, swabs from gills, gill sections and seawater samples from both Farms 1 and 2 were negative for *N. perurans*, as shown in Table 4.3. In Farm 3, only 16.7% (4/24) of gill samples were positive for *N. perurans* by real-time PCR on 30 April 2013. Samples from different fish and sometimes from the same fish on that date were positive to AGD using histology. Combining both analyses, a total of 37.5% (9/24) were positive for AGD (Table 4.3). All real-time PCR analyses were negative in June.

Table 4.3. Prevalence of *Neoparamoeba perurans* on fish and water samples.

Location	Fish	Date	Sample (analysis)	Number	*N. perurans* P (CI)
Farm 1	*O. mykiss*	Nov-11	Gill mucus (PCR)	29	0
	Seawater		1 L seawater	12	0
Farm 2	*S. salar*		Gills (PCR)	20	0
	Seawater		1 L seawater	12	0
Farm 3	*S. salar*	Mar-13	Gill (histology)	15	33 (12-62)
			Gill (PCR)	0	N/A
		Apr-13	Gill (histology)	24	38 (19-59)
			Gill (PCR)	24	
		Jun-13	Gill (histology)	24	0
			Gill (PCR)	24	N/A

P: prevalence, CI: confidence interval, N/A – not available.

4.4. DISCUSSION

The investigation of gill damage on farmed rainbow trout did not show the presence of AGD in November 2011 but the disease could be present on Atlantic salmon at Farm 2 in March 2012 when excess mucus secretion on gills was detected. Other possible causes of gill damage lead to the consideration of other factors such as changes in pH, ammonia, biocides or algal blooms. Gill diseases have been reported as a common problem of trout farms worldwide (Daoust and Ferguson, 1985). The disease has been related to poor water quality due to overcrowding with high levels of fish metabolic products such as ammonia accompanied by low levels of oxygen (Smith and Piper, 1975). Free ammonia is very toxic to fish in freshwater with rising pH and often becomes lethal (Bruno *et al.*, 2013). Very low

levels of undissociated ions of ammonia cause necrosis of gill lamellae, dilated capillaries, hyperaemia, haemorrhage and increased mucus. Nevertheless, controversies have arisen about its effect on fish pathology (Klontz *et al.*, 1985). In an experiment conducted to assess the effect of ammonia in tank-cultured fingerlings of rainbow trout in freshwater, 0.2 mg/L and 0.4 mg/L of un-ionized ammonia were tested for 30, 60 and 90 days. No damage of gill epithelium was observed at the end of the experiment, although some changes in the renal proximal convoluted tubule cells occurred. However, within the first 10 days of the experiment, some neural problems were observed in fish that then recovered and began to feed normally (Klontz *et al.*, 1985). In the present study, the density of rainbow trout were around 3.57- 5.12 kg/m^3 well below the 10 kg/m^3 considered good for fish culture (MD Enrique Madrid, Marine Harvest Experimental Unit, Chinquihue, Chile) and below 14 kg/m^3 accepted as maximum density by the government regulations (Alvial *et al.*, 2012). Atlantic salmon were stocked at 8.16 – 12.41 kg/m^3. The concentration of ammonia determined in 2011 at both farms was below toxic conditions of 0.05 mg/L as well as their related compounds nitrite, nitrate and phosphates (Timmons *et al.*, 2002). In addition, copper compounds are widely used as antifouling agents on nets of cages. Copper leaches from antifouling coatings and could be toxic to fish; however, the concentration of copper in sea water at Farms 1 and 2 was nil or below the detection levels.

Phytoplankton blooms represent a naturally occurring growth of several species that can become a serious problem for cage-farmed fish. The high number of phytoplankton cells may result in the loss of fish through the abrasive action on gills and/or the depletion of dissolved oxygen overnight when they are not photosynthesising. Salmonid requirements for oxygen in the water are 8-10 mg/L as optimum concentration, but show suffocation signs below 3 mg/L (Svobodová *et al.*, 1993). These requirements vary with change in temperature, dioxide carbonate concentration in case of overcrowding, weight of fish (decreasing the requirement when weight increase) and chemical oxygen demand by microorganisms in the water. In the present study only the diatoms were above the normal concentration on two dates for Farm 1 and one date for Farm 2. High numbers of diatoms with moderately silicified frustules such as *Chaetoceros* spp. could irritate gills. These diatoms particularly *C. convolutus* whose spinous setae can penetrate gill tissue and cause physical damage, have been associated with the rapid death of Pacific and Atlantic salmon (Clément and Lembeye, 1993; Bruno *et al.*, 2013). However, it could not have been the case in this present study, because the blooms would be reported promptly by the monitoring service. The other problem caused by

microalgal blooms, the decreased availability of oxygen at night was not reported as a problem at Farms 1 and 2. The oxygen levels of around 8 mg/L were satisfactory for fish respiration (Timmons *et al.*, 2002; Bruno *et al.*, 2013), although there were differences inside and outside the cages, indicating a depletion inside due to fish breathing. In contrast at Farm 3, there was a clear decline of oxygen in autumn with the presence of AGD compared to summer and winter when no AGD was detected, although the concentration near 5.0 mg/L was still above the safe limits for fish considering healthy respiratory epithelium. However, when there is substantial damage to the gill epithelium, as was observed in the present study at Farm 3, this concentration of dissolved oxygen could be critical. This situation could indicate an environmental condition related to AGD and risky environmental sign indicating the probable presence of the disease.

In relation to carbonate hardiness, the low level of kH (6-7) could be explained by the presence of mussel farms that absorb great amounts of calcium carbonate, a required material for their shell construction. This also explains the lower concentration at Farm 1 where mussel cultures were more abundant than at Farm 2. These kH levels, although under the normal value of 8-8.5, are not harmful for fish (Sera GmbH Guides, Germany). These aspects of water quality were not the cause of gill deformity on Farm 1. Other causes that could induce gill problems such as bacteria or virus are reported elsewhere (see Chapter 5).

The environmental factors associated with the development of the clinical AGD disease determined in Tasmania were high sea water temperatures coupled with high salinities, and because the area of historic AGD occurrence on salmon farms was in an estuary at the southeast coast, the retraction of the halocline due to a lower freshwater input from the river (Adams and Nowak, 2003). The outbreaks in Tasmania have been reported at temperatures between 12° and 20°C although the severity of the disease was experimentally reduced at 12-14°C but difficult to control at over 16°C (Zilberg and Munday, 2006). In Tasmania, infections have been correlated with high salinity (32 or more psu) and observed to disappear from fish at salinity 22 psu or less (Zilberg and Munday, 2006). In Chiloé, Chile, the disease was previously detected with temperatures ranging from 9°C to 11.9°C and salinity 33 psu (Bustos *et al.*, 2011). The water salinity in the sea channel at Farm 1 was very stable according to the CTDO monitoring and farm records. Another long-term monitoring across the water column in Farm 1 site between October 2008 and November 2009 also reported salinities ranging between 32.4 - 33.4 psu (Clément *et al.*, 2010). The dissolved oxygen (DO)

was recorded ranging from 5.8 to 12.7 mg/L in the water column for the same area and same period, which lower value was reported to be related to phytoplankton concentration or chlorophyll a values and their photosynthesis activity. The previous study also detected that the Secchi disc variability were mainly related to tidal currents that cause bottom particles re-suspension and a decrease in the water visibility. The lower Secchi disc values at Farm 2 compared to Farm 1 and more phytoplankton at 10 m instead of 1 m indicate less stratification of water column in the former farm. The constriction of the sea channel at Farm 2 compared to the bay shape of the channel at Farm 1 are most probably enabling better water circulation at Farm 2 during changing tides. In relation to water temperatures, the values recorded in the present study at Farms 1 and 2 with the absence of AGD were 10.5-12.5°C, but 11- 13.4°C at Farm 3 with the presence of AGD. This aligned with findings in Tasmania of water temperatures above 10°C as the risk factor for the disease (Clark *et al.*, 2003). In relation to salinity, although there was the proximity of several brooks with probable freshwater discharges at Farm 1 site, the constant oceanic salinity profile did not show the presence of an estuarine area. At Farm 3 where the mouth of Hueihue River was in the proximity, the stable salinity neither showed the presence of an estuarine site. The salinity recorded in the water column at the three farms was very stable at 32.5 and typical of oceanic waters. In Tasmania, AGD was only recorded in the southeast coast where the Huon estuary is present but not in the north and west estuaries of the island. Therefore, estuarine conditions are not a risk factor for the disease but in contrast, could be the source for freshwater bathing treatments for fish. High water temperature and high salinity are also risk factors for AGD, particularly for the seasonal appearance of AGD.

In the reports of AGD in France and Ireland, the disease was related to the higher temperatures of summer and therefore, a seasonal pathology of fish aquaculture (Rodger and McArdle, 1996). Similar patterns were observed in this research. In October and November 1995 in Ireland, AGD was confirmed on eight marine Atlantic salmon farms; although not all of them were experiencing clinical disease such as surface swimming, increase of opercula movements and mortalities (Rodger and McArdle, 1996). The affected farms were in an extended area from the north-west to the south-west on the coastal waters of Ireland, during the warmest summer, highest sea temperatures and scarce rainfalls on records. The disease was also described by Roubal *et al.* (1989) on Atlantic salmon farmed in Tasmania as primarily a summer related problem associated with increasing water temperatures, marine

salinities and the need of freshwater baths to avoid mortalities. AGD reported in this study in March-April 2013 and probably in March 2012, with fish mortalities during sea lice treatments, was more related to these seasonal patterns. Nevertheless, the AGD cases reported in Chile during the ISA epidemic between 2007-2010 (Rozas *et al.*, 2012) corresponded to outbreaks on farms in May, June, August and November 2007 with temperatures as low as 9°C in August (Bustos *et al.*, 2011), in May and June 2008 and July and November 2009 during the colder months of the year in Atlantic salmon, rainbow trout and coho salmon. Therefore, the high water temperature was not always a requirement for an AGD outbreak in this study.

According to the real-time PCR results of gills or water samples, *N. perurans* was not the cause of gill damage in rainbow trout at Farm 1 in November 2011. The gill damage of rainbow trout in 2011 and the excess mucus on Atlantic salmon gills in 2012, five months into the Monitoring and Control Program for *Caligus* could be due to the chemical bath treatments but AGD cannot be ruled out (Fig. 4.2 b). The pesticide deltamethrin has been reported as causing gill damage to catfish *Clarias gariepinus* even at very low concentrations (0.75 – 1.5 μg/L) (Amin and Hashem, 2012). The previous study also reported this pesticide causing oxidative stress and induction of hepatic and kidney damage. In the present study, the concentration used in the bath treatments against sea lice was 0.3 ppm or 300 μg/L. Fish were maintained in the bath for about 40 min indicating the possible short term damage of gills. Although the histological gill sections of the rainbow trout from November 2011 showed hyperplasia of gill epithelium and hypertrophy of mucous cells containing eosinophilic granular substance, the poor fixation of samples prevented this researcher from doing full diagnosis of the lesions and possible causes. In relation to samples at Farm 3 in 2013, the histopathological analysis of Atlantic salmon gills showed the characteristic AGD lesions in March-April according to the description of Adams and Nowak (2004). However, although in June *N. perurans* and AGD were absent, some lesions were observed at the respiratory gill epithelium. In these samples collected one week after the chemical treatment for sea lice, the gill epithelium looked in general normal except for some proliferation of hypertrophic mucous cells with no clear etiology. These lesions were different than the oedematous lesions probably caused by bath treatments described by Godoy *et al.* (2013). It is noteworthy that an increase in mucous cells and the absence of chloride cells in AGD lesions was previously reported (Nowak *et al.*, 2013). In summary, all fish sampled in 2011 and 2013 in this study

showed diverse levels of gill lesions. Therefore, a periodical sea lice treatment with topic chemical baths that could increase gill problems seems inappropriate for fish welfare. The coordinated and periodical treatments initially designed for *Caligus* Monitoring and Control Program did not include topical treatment that can potentially injure gills but in-feed treatments such as ivermectin and emamectin benzoate (Zagmutt-Vergara *et al.*, 2005). The treatments used with the in-feed compounds, only discontinued due to sea lice reduced sensitivity to the compound (Bravo *et al.*, 2008a; Bravo *et al.*, 2014), showed to be better alternatives for an integrated control strategy. The routine chemical bath for sea lice treatment as coordinated integral control strategy should be reviewed by the Chilean regulation (Sernapesca, 2009).

This is the first research that reported a seasonal presence of AGD on farmed salmon in Chile detected by analysis of fish mortality during chemical bath to treat sea lice. Given that the samples of rainbow trout with damaged gill showed no *N. perurans* or other water contaminant, AGD may be confused with other gill diseases without a proper diagnosis. The gill damage on rainbow trout samples and Atlantic salmon that was not related to *N. perurans*, were neither related to water contaminants such as ammonia, diatoms, dinoflagellates and netpen antifouling, these chemical substances were not the cause of gill damage. Chronic gill lesions in all sampled fish indicated a probable cause for sea lice outbreaks and the inappropriate use of chemical bath treatments for the control of these parasites on these farms. Further research on the etiology of these gill pathologies is required as well as a review of the emphasis of coordinated and routine control for sea lice with chemicals. In addition, the probable gill injury caused by chemical bath treatments for sea lice, a secondary effect of the practice, should be investigated in farms as other possible source of gill damage. High temperature coupled with low oxygen concentration in sea water could be a risk environmental condition for fish mortalities during the seasonal appearance of AGD. More *in situ* research with more realistic situations of fish gill disease can be useful for better epidemiological studies of gill damage.

CHAPTER 5 MANAGEMENT OF CALIGIDOSIS ON SALMON FARMS AFFECTED BY *Neoparamoeba perurans*, ISAV AND *Piscirickettsia salmonis* IN CHILE

5.1. INTRODUCTION

Chile was one of the world's major producers of farmed salmon until 2007 (FAO, 2007). The industry was fast growing since its beginning in the mid-1980s (Sernapesca, 1990; 2006) but there was a great impact of diseases since mid-2000s (González and Carvajal, 1994; Johnson *et al.*, 2004; Cottet *et al.*, 2011; Mardones *et al.*, 2011b; Mardones *et al.*, 2014; Rozas and Enríquez, 2014). Sea lice infection is the main parasitic disease that chronically affects the salmon industry in Chile. The present pathogenic species was identified as *Caligus rogercresseyi* by Boxshall and Bravo (2000) as a new species. This fish skin parasite infects all the salmonid species farmed in the marine environment in Chile and some wild fish in the south of Chile (González *et al.*, 2012) and southern Argentina (Bravo *et al.*, 2011). Its native hosts were determined in 1998 (Carvajal *et al.*, 1998; Sepúlveda *et al.*, 2004) as *Eleginops maclovinus* mainly and *Odonthestes regia.* Chemical treatments for sea lice have been applied on Chilean salmon farms since the 1990s without particular regulations (Roth, 2000). Then, the voluntary monitoring and coordinated control programs guided by the salmon farm association was implemented since 2000 (Zagmutt-Vergara *et al.*, 2005) and the compulsory government program in all the industry since 2007 (Molinet *et al.*, 2011). However, the parasite is still a chronic problem with recurrent new outbreaks (Bravo *et al.*, 2013). In 2006-2007 Amoebic Gill Disease (AGD) caused by *Neoparamoeba perurans* was reported concurrent with new outbreaks of sea lice (Bustos *et al.*, 2011). In 2007 the control of sea lice was intensified by the industry in 2007 with chemical baths, due to sea lice resistance shown to the in-feed chemical treatment (Sernapesca, 2007b; a). At the same time, the infectious salmon anaemia (ISA) epidemic occurred (Sernapesca, 2010; Mardones *et al.*, 2011a).

The major ISA outbreaks began in June 2007 along the East coast of Chiloé (Kibenge *et al.*, 2009). ISA is a systemic viral disease of farmed Atlantic salmon without a cure that has been associated with high accumulated mortality reaching 98% (OIE, 2013). The virus had previously been isolated from farmed coho salmon in Chile (Kibenge *et al.*, 2001) and

rainbow trout in Ireland (Mardones *et al.*, 2011a) with no clinical disease. This virus is classified in the new genus *Isavirus*, the first member of the Orthomyxoviridae family characterized in fish, which type's member is the influenza virus (Cottet *et al.*, 2011).

Stress can increase susceptibility of fish to infections. For example, coho salmon with implants of the stress hormone cortisol are more susceptible to sea lice infection (Johnson and Albright, 1992) than salmon which are infected with sea lice as secondary infection. An infection may also predispose fish to another infection. Mustafa *et al.* (2000a) determined that rainbow trout were more susceptible to a second pathogen, the microsporidian *Loma salmonae* when experimentally co-infected with the sea louse *Lepeophtheirus salmonis*. Microscoporidians have been regarded only problematic in the case of immunosuppressed hosts. Recent experimental infections with *C. rogercresseyi* as a secondary pathogen of tank-reared Atlantic salmon demonstrated that this parasite also reduced the resistance of fish to the pathogen *P. salmonis* (Lhorente *et al.*, 2014; Yáñez *et al.*, 2014). The presence of other pathogens that stress fish as well as treatments with routine and inefficient chemical bath would stress farmed fish and could be a risk factor for the copepod infection. In this case, the chronic problem with sea lice infection in Chile could occur because 1) salmon normally resistant to sea lice are more susceptible due to prior-infection by other pathogens and 2) pathogen control strategies used by salmon farms unnecessarily stress the fish. Most of present sea lice control strategies consider sea lice as a reservoir or a vector of salmon pathogens which necessitates their removal. However, this could not be more important than the stressing effect to the host or the stress caused by the mitigation strategies. The aim of this study was to identify the presence of co-infection by other pathogens among farmed salmon with sea lice outbreaks in relation to sea lice control strategies that could explain the chronic sea lice parasitism. This was done as the first step to advise on better and more integrated management and control practices of multiple pathogens in the farms.

5.2. MATERIAL AND METHODS

5.2.1. Farm sites

The same farms and fish sampled in November 2011 and described in Chapter 4, of rainbow trout at Farm 1 and Atlantic salmon at Farm 2 in a sea channel on the eastern coast of the Chiloé archipelago, Chile (Fig. 4.1 a, c) were used for this study. This sampling sites were in the area 10 according to the *Caligus* monitoring and control program (Sernapesca, 2009; Yatabe *et al.*, 2011,Hamilton-West, 2012 #380) where AGD-positive commercial salmon had been identified in 2007-2010 (Bustos et al., 2011). The area is also in the buffer area of the super-spreader farm (where 20% of fish produced the 80% of ISA spread) of the 2007-2008 ISA epidemics (Mardones *et al.*, 2011). In 2013, the same Farm 3 reported in Chapter 4 with Atlantic salmon was sampled (Fig. 4.1 b).

5.2.2. Fish

Fish sampled at Farms 1, 2 and 3 were described in the previous chapter. Only morbid or rejected rainbow trout were allowed for collection at Farm 1 as well as Atlantic salmon at Farm 2 in November 2011. In contrast at Farm 3, only apparently healthy Atlantic salmon were obtained in 2013.

Atlantic salmon were diagnosed with AGD by Pathovet Laboratory (Puerto Montt, Chile) using histology in March 2013 (farm staff, M Vet. A. Santana pers. comm.) during investigation of an increase in the mortality during sea lice treatments. This allowed sampling of fish for diagnosis of multiple pathogen co-infections such as *N. perurans*, ISAV and *Piscirickettsia salmonis.* Histological samples from March were provided by M. Vet. Marco Rozas (Pathovet Laboratory).

5.2.3. Sea louse and fish sampling

Rainbow trout and Atlantic salmon were sampled on 9 November 2011 and only rainbow trout on 18 and 23 November during weekly monitoring for lice. Cage samples were the index cage (in one corner) and two haphazardly selected cages in the middle. The index cages were fixed cages showing higher number of *C. rogercresseyi* during the monitoring. For sea lice counting, the different stages were classified according to the *Caligus* Monitoring and Control Program as ovigerous females (OF), mobile adults, males and non-ovigerous females (A) and chalimus (chalimus 1 to 4). The sum of all the development stages corresponds to the

"total", value that was used in this study. On each fish, the number of sea lice from each development stage was counted and divided by the number of fish sampled to calculate the abundance (Bush *et al.*, 1997). Thirty rainbow trout and 30 Atlantic salmon were collected to count sea lice (Table 5.1). Ten fish were taken from each cage. The fish were netted out and transferred to a bath containing seawater with benzocaine for anaesthesia according to the manufacturer's protocol (BZ 20, Centrovet Chile). After counting sea lice, the fish were returned to their cages if they were not used for organ sampling. For further fish sampling, 29 rainbow trout were obtained during sea lice monitoring on 9 November 2011 on Farm 1 and 20 Atlantic salmon at Farm 2 on 25 November 2011 from two cages and the index cage (Table 5.1). Seven to ten fish were caught from the three cages and placed individually in plastic bags for sampling in the processing room on the seawater site at the sea farm. The index cage at Farm 2 was the nearest cage to the mussel farms (Fig. 4.1c).

At Farms 1 and 2, samples of gills, heart, kidney and liver of Atlantic salmon and rainbow trout were collected and fixed individually in RNAlater or ethanol 99% (Table 5.1). Samples for molecular analysis were frozen at – 20 °C. General gross pathology was observed and recorded by the farm veterinarians, the sampling team and myself. The information was classified according to the salmon farm protocol as mechanical damage (any damage observed to the scales, skin or body), adhesions (connective tissues associated with vaccination that join two structures which would normally be apart (Bruno *et al.*, 2013)), deformity (any body deformity), fish unadapted to sea water (under-sized), piscirickettsiosis (caverns or lesions under the skin as well as liver spots caused by the pathogen *P. salmonis* (see Fryer *et al.*, 1990) and unclassified (organ inflammation by undetermined cause). Other categories were reported in the classification of mortalities throughout the whole culture cycle such as movements (dead during transfer from fresh to sea water), sea lions (predation), elimination (fish collected during sampling for some purpose) and environmental factors (lost to storms and other environmental factors).

In 2013, fish netted out of cages were anaesthetized in a bath containing an overdose of benzocaine (BZ 20, Centrovet Chile). To validate farm data for sea lice counting, sea lice were counted and recorded for each fish (identified as for the research purpose) and compared to the data handed out by the farm to the *Caligus* Monitoring and Control Program (identified as for the survey purpose) (Sernapesca, 2009). Each fish was then placed in a labelled individual plastic bag. A section of the second gill arch was excised and the sample

immediately fixed in 40 mL of Davidson's fixative. For further fish sampling, the bags were transported to the ensilage room next to cages at the sea farm. After about 1 h from fish collection a portion of 10 - 5 mg of gills, equivalent amount of liver and spleen were dissected and placed in RNAlater. Samples for AGD analysis were delivered to Pathovet Laboratory (Puerto Montt), for ISAV to the laboratory at Universidad de Santiago (Santiago) and for *P. salmonis* to ADL Laboratory (PuertoMontt). Then, during the 24-48 h, samples were frozen at -20 °C before processing. Data from the survey purpose were provided by the farm staff. For the survey, four cages were sampled: two index cages in the corners and two haphazardly selected cages in the middle of other cages.

5.2.4. Husbandry

The rainbow trout at Farm 1 were previously subjected to several bath treatments targeting sea lice with the pesticide deltamethrin (0.3 ppm) on 2-7 and 21-24 October 2011 according to the report by the *Caligus* Monitoring and Control Program (Fig. 5.1. b). As not all cages could be simultaneously treated with chemical baths a period of one week was allowed for the treatment of the whole farm. Atlantic salmon at Farm 2 were not treated in October 2011 with chemical baths. Then, after being compelled to do so by the sanitary fisheries authority, coordinate baths between both farms were carried out during the periods of November 4-8 and 19-22 for rainbow trouts and November 10-14 and 25-28 for Atlantic salmon. A different deltamethrin formula was used in November from the formula used in October. The treatments were applied according to the manufacturer's protocol using skirts instead of tarpaulin liners and raising the bottom of the net of salmon cage nets. All rainbow trout were harvested at the end of November at Farm 1 because of gill pathology and poor growth.

The Atlantic salmon at Farm 3 were regularly bath treated against sea lice with the pesticide cypermethrin (0.3 ppm/m3) in 2013 (Fig. 5.2 b). Since March to June 2013 (Fig. 5.2. b) some but not all the cages were bathed every week. Only in the first week of April (week 14), the chemical deltamethrin (0.3 ppm) was added to the previous bath treatments (M Vet. J. Gatica pers. comm.) and all the cages were treated only once. Then, the regular but partial bath treatments with cypermethrin were resumed each week except for some periods of time and during the sampling in April for this study (M Vet. J. Gatica pers. comm.). In May bath treatments with cypermethrin were only applied the last two weeks (one out of 4 cages on week 20, 3 out of 4 cages in week 22). In June, 3 out of 4 cages were treated only on weeks 23 and 24. Bathing treatments were done according to the manufacturer's protocol and again

using skirts instead of tarpaulin liners so that the product could diffuse to sea water. Gill sampling for this year was done during a week when no sea lice treatment was performed. After June, bath treatment was not required according to the *Caligus* Monitoring and Control Program due to the low sea water temperature. The average temperature at 5 m depth was 11.78 ± 0.12°C in April, 10.99 ± 0.02°C in May and then dropped to 10.55 ± 0.04°C in June and 10.08 ± 0.04°C in July.

5.2.5. Histopathology and real-time PCR for *N. perurans*

The data of AGD lesions and *N. perurans* detection using real-time PCR from the fish sampled at Farms 1, 2 and 3 were obtained from the previous chapter.

Table 5.1. Number of fish, parasites and organ sampled for *Caligus rogercresseyi*, *Neoparamoeba perurans* or AGD, ISAV and *Piscirickettsia salmonis* identification at Farms 1, 2 and 3. K: kidney, L: liver, H: heart, Number: number of samples.

Location	Fish	Date	Sample (analysis)	Number
Farm 1	*O. mykiss*	Nov-11	Fish for sea lice	30
			Fish gill mucus	29
			K	29
Farm 2	*S. salar*		Fish for sea lice	30
			Fish gills	20
			K/L/H	20
Farm 3	*S. salar*	Mar-13	Fish for sea lice	40
			Gills (histology)	15
			Gills (PCR)	0
			K/L/H	0
		Apr-13	Fish for sea lice	40
			Gills (histology)	24
			Gills (PCR)	24
			K/L/H	24
		Jun-13	Fish for sea lice	40
			Gills (histology)	24
			Gills (PCR)	24
			K/L/H	23

5.2.6. Molecular analysis

5.2.6.1. Total nucleic acid extraction

The total nucleic acid (TNA) extracted from gill samples and gill swabs in the previous study (see Chapter 4) were used in the present study as well as the TNA extracted from kidney, liver and heart (Table 5.1). The samples were also processed with the MasterPure™ Complete DNA and RNA Extraction Kit (Epicentre) for nucleic acid extraction according to the manufacturer's protocol. The DNA and RNA pellets were resuspended in 40 to 100 µL of nuclease free water.

Ribonucleic acid was extracted for ISAV analysis from 5-10 mg of gills of the 29 rainbow trout and gills, heart and liver of the 20 Atlantic salmon using an E.Z.N.A. total kit I according to manufacturer's protocol (Omega Bio-tek). The samples were then applied to a HiBind RNA spin column placed into a 2 mL and centrifuged at 10,000 x ***g*** for 60 s. The RNA collected in the column was washed twice by adding 500 µL of RNA Wash Buffer II and centrifuging at 10,000 x ***g*** for 30 s and dried by centrifuging only. The RNA sample was finally collected from the column eluting with 40-70 µL of DEPC treated water and centrifuged at 13,000 x *g* for 2 min.

Extraction of DNA and RNA from samples was done at Universidad de Santiago as described and subsamples as backup were sent to Pathovet and ADL Laboratories (Puerto Montt, Chile) for the identification of *N. perurans* and *P. salmonis*.

5.2.6.2. Conventional and real-time reverse-transcription PCR (real-time RT-PCR) test for ISAV

Gill samples were analysed for the presence of ISAV nucleic acids by real-time RT-PCR for segment 8 of the virus according to the method described by Cottet *et al.* (2010) with modifications. The positive control was this supernatant of cell culture infected with ISAV 901 kept at the Laboratorio de Virología, Universidad de Santiago, Chile. The TNA of 300 µL of the supernatant was extracted using the MasterPure™ extraction kit and eluted in 40 µL of DEPC water from which 2 µL were used as positive control. The chemistry used was a SensiMix SYBR 1 step test in a master mix containing: 10 µl of 2x SensiMix One step, 1 µL of primers F5 (5´-GAAGAGTCAGGATGCCAAGACG-3´) 10µM, 1 µL of R5 primer (5´-

GAAGTCGATGATCTGCAGCGA-3´) 10µM, 0.4 µL of Ribosafe RNAse inhibitor, a minimum of 2 µL of RNA sample and DEPC treated water to adjust a total volume of 20 µL reaction. The reactions were read in the same Stratagene Mx3000P real-time PCR Systems with SYBR Green and Roxy as dyes. The thermal protocol used in 2011 was 20 min at 42°C and 10 min at 95°C; 39 cycles of 15 s at 95°C, 15 s at 60°C and 15 s at 72°C; and a melt curve of 1 min at 95°C, 30 s at 55°C and 30 s at 95°C. The thermal profile used in 2013 was 30 min at 50ºC and 2 min at 94ºC, followed by 39 cycles of 15 s at 94ºC, 30 s at 68ºC, with a final extension of 5 min at 68ºC.

Two-step RT-PCR was performed on the total RNA extracted from heart and liver samples to detect ISAV segment 8. For the ISAV positive samples, an amplification of the HPR region of segment 6 that encoded the haemagglutinin-esterase was performed to identify the virus genotype. For reverse transcription, the cDNA was obtained using the M-MLV Reverse Transcriptase (New England Biolabs) incubating the reaction mixture for an hour at 37°C. For the PCR, the master mix to adjust a total volume of 50 µL for the reaction contained: 5 µl of 10x buffer, 0.4 µL of dNTPs 25mM, 2.5 µL of primers F5 (5'-GAAGAGTCAGGATGCCAAGACG-3') or Vir F1 (5'- AAGCAACAGACAGGCTCGAT-3') 10µM, 2.5 µL of R5 (5'-GAAGTCGATGATCTGCAGCGA-3') or Vir R1 (5'-AAGCAACAGACAGGCTCGAT- 3') primers 10µM, 34.1 µL of DEPC treated water, 0. 5 µL Paq 5U/µL and 5 µL of cDNA obtained in the RT step. These primers yield a PCR product of 220 bp. The samples were amplified twice to obtain sufficient DNA products for cloning. The cycler reaction was as follow: 94°C for 5 min (1 cycle); followed by 35 cycles of the sequence 94°C for 30 s, 54°C for 30 s, 72°c for 30 s; ending by 72°C for 7 min. PCR were performed in a PTC-100 Peltier Effect Cycler (MJ Research, Inc).

The PCR products for the segment were electrophoresed in 2% agarose gel in TAE buffer and visualized under UV light after staining with ethidium bromide. The positives samples for amplification using primers VIR was amplified using high fidelity enzyme Platinum Pfx DNA Polymerase (Invitrogen) according to the manufacturer's protocol. The positive samples for these primers were purified from the gel using the extraction kit Wizard SV gel and PCR Clean-Up System (Promega). The PCR product was cloned in pGEMT Easy (Promega) according to the manufacturer´s instructions for subsequent sequencing. Plasmid DNA segments were sent for sequencing of both strands (Macrogen, South Korea). The bioinformatic analysis was performed with Chromas Lite and Protein Sequence Analysis V

3.31.3. Homologue sequences were determined using BLAST software (Altschul *et al.*, 1990) from a database deposited in GeneBank as previously described (Cottet et al. 2010).

5.2.6.3. Conventional and real-time PCR to detect *Piscirickettsia salmonis*

The presence of *P. salmonis* in the samples was investigated using single-step conventional PCR to amplify the internal transcribed spacer (ITS) of the rDNA of the ribosomal operon. The primers used were RTS1 (5'-TGA TTT TAT TGT TTA GTG AGA ATG A -3', forward F-223), RTS2 (5'-AAATAACCCTAAATTAATCAAGGA-3'; reverser R-266), and RTS4 (5'-ATG CAC TTA TTC ACT TGA TCA TA- 3', reverse R-459) previously described by Marshall et al. (1998). The cycler reaction was 95°C for 3 min (1 cycle), followed by 36 cycles at 94°C for 15 s, 50°C for 30 s, 72°c for 30 s; and 72°C for 7 min. The expected lengths of the reaction product were 283 bp with primers RTS1-RTS4 and 91 bp for RTS1-RTS2. Conventional PCR was performed in a PTC-100 thermocycler Peltier-effect cycler (MJ Research Inc.) and amplicons analysed by electrophoresis in 2% agarose gels in 1x Lithium Borate buffer and stained with GelRed (1µmL/10 ml). Sub-samples were analysed by ADL Diagnostic Laboratory (Puerto Montt, Chile) using real-time PCR in a Light Cycler S480 (Roche Diagnostics, Germany). Real-time PCR was carried out using a DNA Master Hydrolysis Probe Kit (Roche) together with primers and probes. A master mix with a total volume of 10 µL was prepared containing 0.3 µM Taqman probes, 0.9 µM of primers and template DNA.

5.2.7. Statistical analysis

The numbers of sea lice recorded per fish were analysed using log + 1 transformed data and a nested analysis of variance as well as Chi-Square analysis of the Generalized Linear Models (SPSS Inc. 21.0, Chicago, USA). Results were considered significant at $p<0.05$ for rejection of the null hypothesis of similarity of groups.

The abundance of sea lice were compared between dates with presence/absence of AGD (results from Chapter 4), ISAV and *P. salmonis* using Chi-Square from the Generalized Linear Models.

5.3. RESULTS

5.3.1. Sea lice

The sea lice burden on fish at Farms 1 and 2 is shown in Fig. 5.1. Adult and chalimus stages of *C .rogercresseyi* were present on all farmed *O. mykiss* in the November sampling that was done one week after treatment for sea lice. The prevalence of sea lice within the rainbow trout and Atlantic salmon sampled was 100% in the three farms. Sea lice abundance was higher on the Atlantic salmon at Farm 2 which had not been treated with chaemoterapeutants, than on the rainbow trout at Farm 1 that had been treated with the first deltamethrin compound (Fig. 5.1 a). The analysis of variance showed a significantly larger number of all the sea louse stages, OF ($F_{4, 54}$: 8.93, $p<0.001$), A ($F_{4, 54}$: 5.749, $p=0.001$) and Ch, ($F_{4, 54}$: 4.172, $p=0.005$) on the Atlantic salmon than on the rainbow trout.

The number of total sea lice was significantly higher (abundance ± standard error: 44 ± 16) on rainbow trout at the beginning of November than at the beginning of October even though the fish were treated twice in October (Fig. 5.1 b). The treatment was changed to a bath with a new formula of deltamethrin every 15 days in November 2011. This reduced the population but did not eliminate the parasites. Treatment resulted in a significantly lower abundance for all stages at the end of November (Ch, OF $F_{1,158}$: 133.6, $p<0.001$, A $F_{1,158}$: 133.4, $p<0.001$, Total F1, 158: 72.6, $p<0.001$). Nevertheless, the abundance of Ch was 10 ± 2, of OF 2 ± 1 and A (males and non-ovigerous females) 3 ± 1. As the number of total adults was less than 6, the fish did not require further treatments according to the regulations (Sernapesca, 2012) although the total number was 15 ± 3 parasites per fish. However, all rainbow trout were harvested at the end of November due to poor growth, gill disease and sea lice.

On 30 April 2013 at Farm 3, a lower abundance and standard error of total sea lice of 3 ± 0.6 per fish were found compared to 19 June when 10 ± 1.8 were observed. In June when there was no treatment and no AGD (see below) not only the abundance of sea lice was greater but also the range was greater (range 5-25 in June and 1-9 in April). Sea lice counting for the research and survey purpose showed abundances ranging from 3 (2-4.2) of total sea lice on 5 May to 8 (7-15.2) on 19 June (Fig 5.2 a). Comparing data from April-May to June, a significant higher abundance of total number of sea lice were observed for the research (Chi.-square 150.74 df 1 $p< 0.0001$) and survey (Chi-Square:130.08 df 1 $p<0.0001$) in June. The Chi-square analysis to compare the distribution of total sea lice between the research and

survey purpose samplings showed no differences between the sampling data on 30 April to 5 May (Chi-Square: 0.266, df 1 N: 20 p=0.606) and on 19 June to 20 June (Chi-Square: 0.194, df 1, N: 20, p=0.660) validating the use of data obtained from the Monitoring program.

Taking into account all the data at Farm 3 for the research and the survey, *S. salar* sampled during March to June 2013 always showed the presence of adult and chalimus stages, although periodical treatments for sea lice (almost every week but only some cages) were applied with cypermethrin (Fig. 5.2 b). Nevertheless, the maximum abundance of sea lice was always lower at Farm 3 (12) than the maximum abundance at Farm 1 (70) and Farm 2 (43). A real reduction of sea lice (but not eradication) was only possible when all the cages were bathed with deltamethrin in April week 14 at Farm 3 (n: 40, median 3, Chi-Square 52.0, df 1, $p<0.001$) and AGD was present. The abundance of total sea lice was in general higher during the overall time of the study during the weeks with no treatments. The total sea lice abundance on Atlantic salmon treated with cypermethrin bath (n: 370, median 5) was lower than the abundance of sea lice on no treated fish (n: 270, median 7) (Chi-Square 143.7, df 1, $p<0.001$).

5.3.2. Macroscopic clinical signs

The most notable problem with the rainbow trout was gross deformity of the gills, with haemorrhage, excess mucus and sometimes shortened operculum (Fig. 4.2 a). This condition was not due to *N. perurans* according to the results in Chapter 4. At Farm 2, gills of Atlantic salmon appeared normal with no signs of mucous spots or patches (figure not shown) but abnormal mucous gills were reported by the veterinarian in March 2012 (Fig. 4.2 b). The causes of mortalities sampled on 15 November at Farm 2 according to gross pathology signs, are shown in Fig. 5.3. One week after beginning with chemical bath treatments the most important cause was mechanical damage (45.5% of total Atlantic salmon mortalities), while 12.2% was due to piscirickettsiosis as indicated by the presence of caverns or white spot on the liver both signs of *Piscirickettsia salmonis* infection (OIE, 2006) and 13.6% due to adhesions (Bruno *et al.*, 2013). During the whole growing cycle, the proportion of fish dead due to these causes were 12%, 7% and 32% respectively of the total number of dead fish.

There was no sign of gross gill changes such as raised, white mucoid spots or patches typical of AGD in April or June 2013. Neither there were signs of pale gills characteristics of ISA. In

June one small black male was between the rejected fish. The other fish collected all females as expected, had dark skin and showed early gonad development. Also in this sampling, two cages that were in the middle of the southern group of cages were reported to suffer mortalities due to piscirickettsiosis on the basis of gross signs. Two other cages corresponding to the edges of netpens had the higher burden of sea lice. Four fish from mortalities observed with positive signs for piscirickettsiosis came from one of the cages in the middle and two from the cages in the edges (Fig. 4.1 b). The routine farm monitoring and recording of month accumulated mortalities determined that 2% of fish dead died by piscirickettsiosis in April and 12% in June. The corresponding mortalities due to mechanical lesions were 2.5% (144 fish out of 5769 total fish mortalities) in April and 15% (268 fish out of 1790 total fish mortalities) in June.

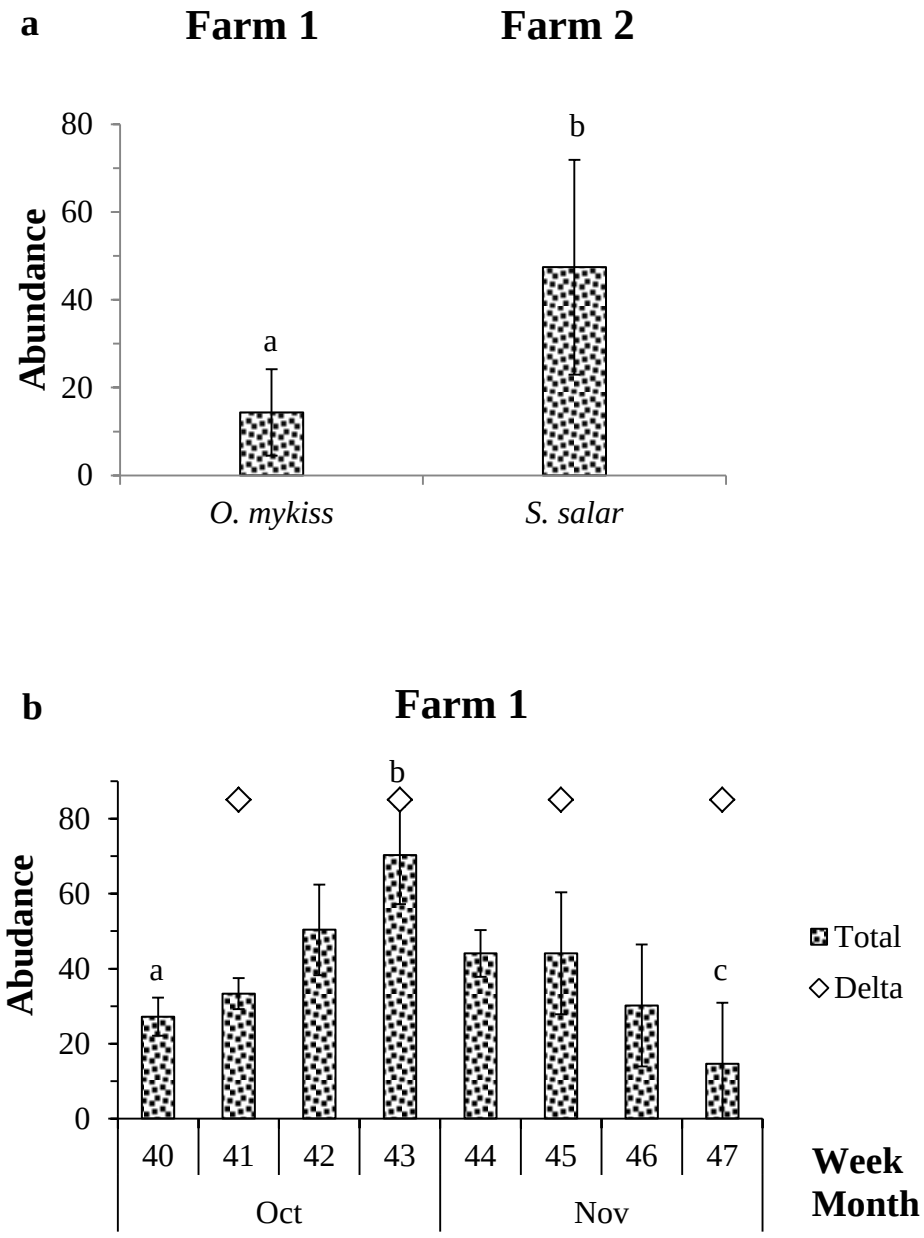


Figure 5.1. a) Abundance and standard error of Total stages of *Caligus rogercresseyi* on *O. mykiss* at Farm 1 compared to *S. salar* at Farm 2 on 9 November 2011; b) abundance and standard error of total stages of *C. rogercresseyi* on *O. mykiss* during the monitoring and treatment program in November 2011 at Farm 1; the treatment dates are indicated by rhombus on the top. Total: total number of sea lice of all the development stages. Delta: dates when cages were treated with deltamethrin. Letters a, b and c indicate significant different abundance of sea lice. The other dates were not statistically compared.

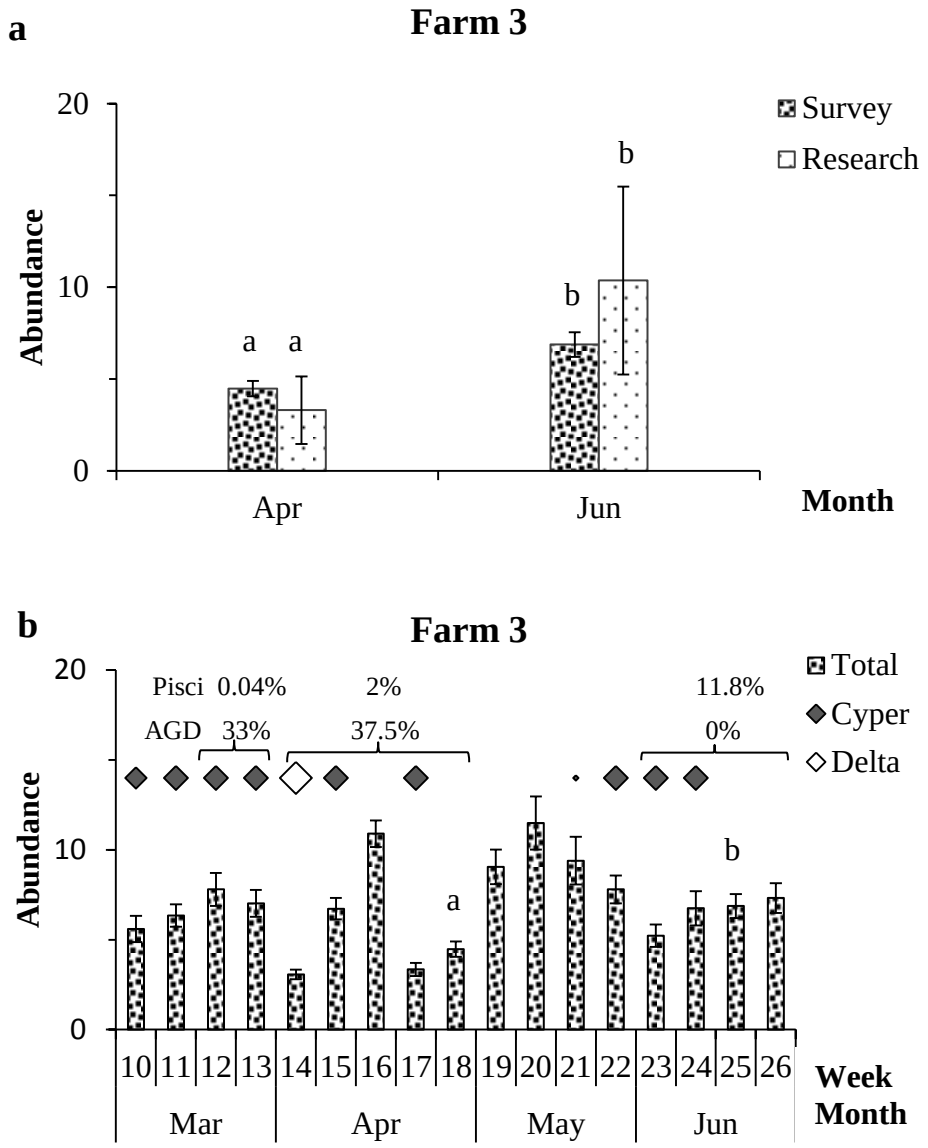
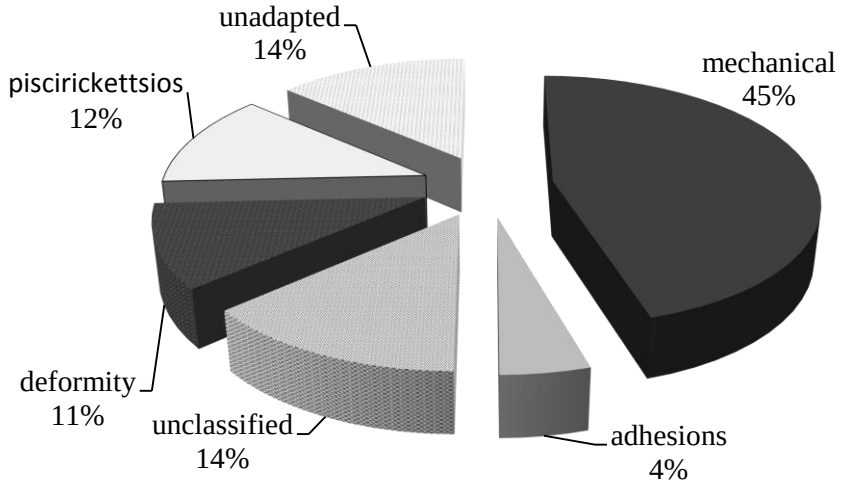


Figure 5.2. a) Abundance and standard error of total sea lice for the research purpose compared to the survey purpose on April and June 2013 at Farm 3; b) abundance and standard errors of total sea lice on Atlantic salmon at Farm 3 from March to June 2013 according to *Caligus* Monitoring program; date of bath treatments for sea lice with cypermethrin (Cyper) and deltamethrin (Delta) are indicated. Because not all the cages were bath treated, shorter or larger rhombi for Cyper corresponded to the treatment of one or three of the four cages sampled for sea lice. Pisci corresponds to the proportion of total mortalities due to piscirickettsiosis in March, April and June; and AGD, to the proportion of fish with this disease in the samples. The large rhombus for deltamethrin indicates that all cages were treated. Letters a, b, c, d and e indicate significant different abundance of sea lice between these samples.

a) **Cause of fish mortality on 15 November in the farm**



b) **Total mortalities throughout the growing cycle in the whole farm**
(% of fish in culture, % of mortalities)

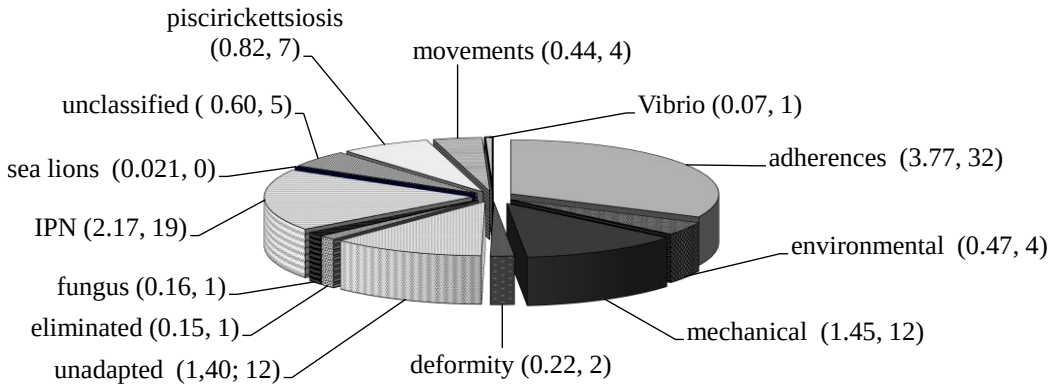


Figure 5.3. a) Fish gross pathology classification of *S. salar* at Farm 2 for the mortalities sampled on 15 November and b) mortality classification for the whole growing cycle in the farm. Mechanical: mortality due to mechanical damages; unclassified: inflamed organs without a clear cause; deformity: deformed fish; piscirickettsiosis: presence of caverns, liver w/spots; unadapted: small fish; adhesions: filaments joining organs; movements: dead during transport from fresh to sea water; sea lions: dead by predation; eliminated: sampling for some purpose; environmental: lost to storms and other factors.

5.3.3. Gill histology for AGD and real-time PCR analysis for *N. perurans*

Taking into account all the histological and real-time PCR analyses (see Chapter 4), only sections from gills of Atlantic salmon collected on 11 March 2013 at Farm 3 showed 33% (5/15) prevalence for AGD and on 30 April, a total of 37.5%(9/24) prevalence for AGD and *N. perurans* (Fig. 5.4). In June, AGD and *N perurans* were not detected in any samples.

5.3.4. Molecular analysis of samples

At Farm 1 in November 2011, 17% (5/29) of rainbow trout gills were positive for ISAV segment 8. On Farm 2 30% (6/20) of gills and 80% (16/20) of liver and kidney of Atlantic salmon were positive for ISAV segment 8 (Fig. 5.4). It was possible to amplify the HPR region of segment 6 of ISAV from only two Atlantic salmon initially positive for ISAV segment 8. The sequence of the amplicons of the HPR region allowed the identification of the viral genotype strain. The sequences aligned in BLAST program and compared to the reported RNA sequences for HPR region of segment 6 showed that the sequences matched those under accession number GU830900-1 of the Chilean isolate ISAV752_09 of GenBank (http://www.ncbi.nlm.nih.gov/genbank). The sequences determined from the two cDNA clones are similar in a 99% to the full length already sequenced RNA segment of isolate ISAV752_09 that displays HPR 7b genotype. The alignment shows that this ISAV HPR sequence corresponds to the HPR 7b with two site-specific mutations. This is one of the already described and most common of Chilean virus associated with outbreaks of high mortality rates in Chile starting in June 2007 to November 2008.

On 30 April 2013 at Farm 3, 33.3% (8/24) of kidney, liver and spleen of Atlantic salmon were positive for segment 8. In June only a 21.7% (5/23) of samples from fish kidney, liver and spleen (K/L/S) were positives for the virus. Because the re-amplification run on samples for ISAV gave positive result but no mortality showing ISA clinical signs was observed, the samples were considered sub-clinically positive for segment 8. It was possible to amplify the HPR region of segment 6 of ISAV from two samples of Atlantic salmon from April and three from June 2013. In April one sample corresponded to ISAV 901 with HPR 1c and one positive for ISAV 752 with HPR 7b. In June, two samples were positive for ISAV 901 and one sample for ISAV 752. The sequences aligned in BLAST program and the specificity of the oligonucleotide annealing sites determined a 98-99% similarity with the previous reported

segment under accession number GU830908-1 of the Chilean isolate ISAV901 in GenBank with no site-specific mutations, and with the isolate under accession number GU830900.1 length 1167 bp of the Chilean ISAV 752 with no mutations.

In relation to piscirickettsiosis, in 2011 samples 28% (8/29) of kidney samples in rainbow trout and 10% (2/20) of pooled samples prepared from samples of liver and heart in Atlantic salmon were positive for *P. salmonis* using the real-time PCR reactions (Fig. 5.4). This was in agreement with the second most important cause of daily mortality, which was 12.2% of piscirickettsiosis according to the gross pathology checking on Atlantic salmon dead in one day in Farm 2 (Fig. 5.3 a). None of the 2013 April and June K/L/S samples, corresponding to the apparently normal and actively swimming fish, were positive for *P. salmonis*. Nevertheless, according to the farm monitoring records of accumulated fish mortalities for each month there were 0.04% (10/26467) mortalities due to piscirickettsiosis in March, 2.3% (134/5769) in April and 11.8% (268/1790) in June at the whole farm (M. Vet. J. Gatica, pers. comm.).

Increase of fish mortality due to piscirickettsiosis was thus observed in June when AGD was not present compared to April when AGD was present in fish. Nevertheless, there was no significant difference in prevalence of ISAV between June when AGD was absent compared to April (Chi-Square: 0.188, df 1, N: 47, p=0.665) when AGD was present.

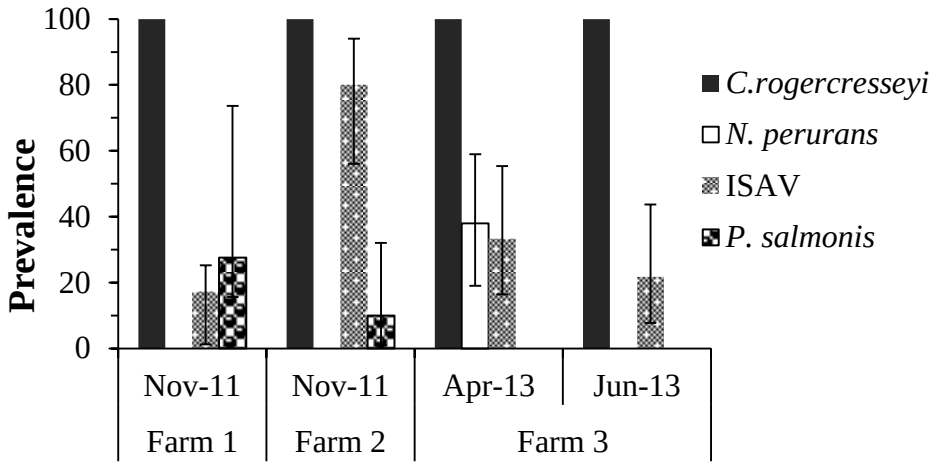


Figure 5.4. Prevalence of infected fish with *Caligus rogercresseyi*, *Neoparamoeba perurans* and AGD, ISAV and *Piscirickettsia salmonis* on morbid *O. mykiss* at Farm 1, morbid *S. salar* at Farm 2 and apparently healthy *S. salar* at Farm 3.

5.4. DISCUSSION

The most prevalent pathogen after sea lice in the 2011 samples was *P. salmonis* in particular at Farm 1 where 28% of rainbow trout were affected by piscirickettsiosis. Ten percent of Atlantic salmon at Farm 2 was positive to this pathogen. It is noteworthy that these samples were from morbid fish. This pathogen was absent from the apparently healthy and actively swimming fish sampled in April and June 2013 at Farm 3. Nevertheless, in this farm the routine farm monitoring of mortalities based on gross sings determined that 0.04%, 2.8 % and 11.8% of fish died from an infection with *P. salmonis* in March, April and June respectively. The reduction observed in March and April occurred when AGD outbreaks were present. The presence or absence of AGD in 2013 in this study did not seem to affect the prevalence of ISAV as it was similar in April and June.

Piscirickettsiosis in fish was first recognized in Chile and remains one of the major diseases affecting Chilean salmon aquaculture in marine and estuarine waters. Epizootics occurred in all salmonid species farmed in Chile, Atlantic salmon *Salmo salar*, Chinook salmon *O tschawytscha*, masou salmon *O. masou* and rainbow trout *O. mykiss* (Rozas and Enríquez, 2014). The disease is a chronic problem in Chilean salmon aquaculture because of the lack of efficient and commercial antibiotics (Cabello, 2006) and vaccines (Marshall *et al.*, 2007; Tobar *et al.*, 2011). Although *P. salmonis* is sensitive to antibiotics *in vitro*, infected fish do not respond in the same way to this treatment (Rozas and Enríquez, 2014). In addition, vaccines cause adhesions as a side effect, a pathology condition resulting in death by digestive tract constriction (Bruno *et al.*, 2013). The present study found a high percentage of mortality in Atlantic salmon due to adhesions and the first cause of mortality in the accumulated mortalities monitoring. The presence of *P. salmonis* in both fish species and the vaccines applied, in addition to the gill lesions of unknown aetiology, probably indicate the most important co-infectious pathogen with sea lice that should not be treated as primary and single pathogen.

The presence or absence of AGD in 2013 in this study did not seem to affect the abundance of sea lice. The results in the present study showed a reduction of sea lice in April 2013 on AGD affected fish, but with integral and coordinated chemical baths to treat the parasite compared to the fish in June without AGD and without chemical baths. Considering only sea lice, a higher abundance of the copepod should be expected in April compared to June due to

water temperature but distortions of data were observed. The lower water temperature observed in this study in June compared to April (see Chapter 4) should reduce the length of the copepod's generation time and therefore, reduce their reproduction and the release of infective larvae (González and Carvajal, 2003). If *P. salmonis* was the co-infection causing the rise in sea lice abundance and mortalities caused by *P. salmonis* was reduced when AGD was present, a lower abundance of sea lice would be expected. As fish mortalities were observed only during bath treatments and AGD outbreaks, this could indicate that the mortalities were related to these baths concurrent with gill lesions. The pesticide deltamethrin has been reported as causing gill damage as well as hepatic and kidney damages to catfish *Clarias gariepinus* (Amin and Hashem, 2012). Therefore, routine bath with this chemical and cypermethrin to treat sea lice seems inappropriate in case of AGD affected fish with extensive hypertrophic lesions on gill filaments. Sea lice parasitism with *L. salmonis* on farmed salmonid has been associated with lethal and sub-lethal effects that include fish stress particularly with pre-adults and adults stages (Johnson and Albright, 1992; Mustafa *et al.*, 2000b) as well as depression of the immune system and skin damage of fish (Costello, 2006; Fast, 2014). In addition in this study, as the bath treatments did not effectively eradicate the parasite, recurrent chemical baths to treat sea lice were used. This was also observed in previous studies where fish were treated for longer periods of time to increase the compound efficacy (Zagmutt-Vergara *et al.*, 2005; Bravo *et al.*, 2014). Repeated chemical baths could cause an increase of host stress and reduction in their immune response. With existing gill lesions, particularly in the case of rainbow trout at Farm 1, chemical bath treatments did not solve sea lice problem which at the end led to the early harvest of the fish due to disease problems and poor growth. Further temporal studies on the dynamic of sea lice abundance would be interesting to see the relationship with the presence/absence of AGD and without chemical treatments.

The growing abundance of sea lice on rainbow trout in October 2011 suggests a fish population that have not been treated, although the farm staff reported that they treated with deltamethrin parasiticide every 15 days. This could be due to the lack of delousing coordination measures between farms sharing the same neighbourhood area. This was also described by Bravo *et al.* (2013) as one of the major influence on sea lice abundance on farms. Although this abundance could be due to the reduced sensitivity of lice to the compound (Helgesen *et al.*, 2014), the reduction of sea lice in November after treatment with

a new deltamethrin brand indicates no resistance of the copepods to the pesticide that occur with recurrent treatments with the same product (FAO, 2004). However, although the use of these bath treatments with deltamethrin in November reduced the number of sea lice, it still did not eliminate them. Salmon farmers did not bath Atlantic salmon against sea lice at Farm 2 in November in spite of regulations that make compulsory the coordinated treatment of the parasitism in salmon farm areas when its abundance was over 6 adults per fish (Sernapesca, 2009; González *et al.*, 2015). One of the reasons for doing this could be to prevent sea lice resistance to the few effective chemicals available as previously observed (Bravo *et al.*, 2008a; Yatabe *et al.*, 2011; Helgesen *et al.*, 2014) and/or to avoid fish mortality due the presence of co-infection with other pathogens or other pathologies (Lhorente *et al.*, 2014; Yáñez *et al.*, 2014). The topical treatments most used for sea lice are deltamethrin and cypermethrin (Sernapesca, 2013). In-feed treatments include emamectin benzoate (use discontinued since 2008 in most Chileans farms) and diflubenzuron, which have shown debatable efficacy lately, and azametiphos the last generation of organophosphates used as bath treatments (Bravo *et al.*, 2008a; Sernapesca, 2009; 2013). One of the problems solved with the in-feed treatment was that simultaneous treatments of a whole farm and neighbouring farms were possible due to logistic. But as only emamectin benzoate was effective to treat all the sea lice stages, this compound was approved as single chemical against sea lice from 2000 to 2007. As a consequence, the reduced sensitivity of *Caligus rogercresseyi* to emamectin benzoate was reported by SalmonChile that claimed for the need for new compounds to be approved (Sernapesca, 2007b; Bravo *et al.*, 2008a). The alternative and technically available compounds that were legalised by the authority in Chile were a return to the previous strategy of chemical baths against sea lice that take at least a week or more (in case of climatic problems) for a coordinated treatment of all the cages in a farm or neighbouring farms. The real coordination of bath treatments as requested by the national regulations for and integrated pest management of sea lice (Sernapesca, 2009; Bravo *et al.*, 2013) is not possible with chemical baths due to logistics. This could be the cause of cross-infection and later multiplication of sea lice on fish from cages that have not been treated yet to the cages already treated in same or neighbour farms, which could explain the chronic presence of the parasite.

Although persistence of sea lice on rainbow trout after treatments could be due to residual parasites from the same farm, they could also result from cross-infection by larval and adult

C. rogercresseyi from the un-treated Atlantic salmon farm. Studies of *L. salmonis* parasite of farmed salmon in the northern hemisphere have shown that sea lice are less abundant on fish if farms are more than 10 km apart (Tully, 1989; Costello, 2006). Larval dispersal from sea lice of farmed salmon can reach 30 km depending on tidal currents and prevailing winds (Middlemas *et al.*, 2013). In the present study, the distance between farms was only 2.7 km and within a sea channel where the main currents were related to tides (Sievers and Silva, 2008). Another study in Chile also suggested that the distance between farms in the same management area was insufficient (Molinet *et al.*, 2011). This latter study that investigated the temporal and spatial dynamic of copepodid stages of *C. rogercresseyi* compared to water circulation patterns in an embayment, where eight intensive salmon farms were located, showed cross-infestation by sea lice although they were following the regulations implemented by the Chilean Fisheries Law for salmon aquaculture (Molinet *et al.*, 2011). These results indicate that the minimum distance between working farms of 1.5 miles or 2.778 km (Alvial *et al.*, 2012) should be reviewed in Chile. Previous studies of farmed salmon under field conditions and experimental infection with *C. rogercresseyi* determined that *O. mykiss* were most heavily colonized by the copepod compared to *O. kisutch* and *S. salar* when naïve fish with similar weight were used (González *et al.*, 2000). *O. kisutch* was the most resistant species with lower prevalence and abundance of the parasite, as well as showing no record of reproductive females. Thus, with Atlantic salmon being reared at Farm 2, rainbow trout is the least suitable species to rise next to them considering that the farms were not far enough from each other to avoid cross-infection. In those previous studies, *O. kisutch* was advised as a better species for sites with sea lice problems such as Farm 1 considering only sea lice (González *et al.*, 2000). But in case of a co-infection such as piscirickettsiosis, the resistance of *O. kisutch* to the parasite was similar to *O. mykiss* (González *et al.*, 2000). Therefore, Farm 1 and Farm 2 should be considered as the same farm and populated with the same species or be fallowed.

The coordination of sea lice treatments between Farm 1 and 2 that should have been occurring according to the sea lice control program (Sernapesca, 2009) was also a problem considering the presence of a pathogenic strain of ISAV in the internal organs of Atlantic salmon. ISA is primarily a disease of Atlantic salmon and has been causing great losses in Chilean salmon aquaculture (Mardones *et al.*, 2011a). The presence of ISAV in gills of rainbow trout that do not affect this species could suggest a host infection by the virus and/or

the presence of the virus in sea water. Rainbow trout had been experimentally infected with ISAV to prove that this species can act as carriers (Rimstad *et al.*, 2011). The presence of the virus in the 2011 samples should not seem so odd considering that Farms 1 and 2 were in the control area where 2007 ISA outbreak began (Kibenge *et al.*, 2009). The present study indicated a probable chronic presence of ISAV on farmed fish in the area sampled, although at subclinical level. The Chilean isolates related to the previous epidemic outbreaks were ISAV752_09 and ISAV901_09 (Cottet *et al.*, 2010). Different strains of ISAV have diverse virulence such as highly virulent, slightly virulent and ISAV avirulent and a non-culturable strain. It has been widely accepted that the level of virulence of the virus is related to the High Polymorphic Region (HPR) type from the RNA segment 6 of the virus, with the larger one being the HPR0 which is the avirulent strain (Cottet *et al*., 2010). Nevertheless, it has been observed that sometimes the virulent strains can appear as avirulent types as was the case in the present study. Therefore, it has also been suggested that pathogenicity is the consequence of multiple factors rather than the HPR region as the only virulence marker (Cottet *et al.*, 2010). In addition, it has been proposed that mutations of the polymorphic complex could be an adaptation to the environment (i.e. temperature or host susceptibility), allowing its wide distribution along the Chilean coast. The ISAV 752_09 isolate was reported to have a prevalence of 80% (Kibenge *et al.*, 2009). At present according to Godoy *et al.* (2013) most of the samples collected by the compulsory and national ISAV monitoring program in the area appeared to be replaced by the low pathogenic HPRO and sometimes appearing some isolated outbreaks of pathogenic variants. In this study instead, the pathogenic ISAV 752_09 and 901_09 with HPR 7b and 1c respectively were present at subclinical levels in the 2011 and 2013 samples and no the HPR0. It is possible therefore, that the effect of a pathogenic strain on the fish from low pathogenicity to another more aggressive one can be avoided by reducing fish stress, for instance in this case, by reducing the excessive chemical baths against sea lice.

This is the first integrated and field study of the diseases of farmed salmon to advise a holistic control strategy. The study indicated *P. salmonis* the most important co-infection with sea lice. But a reduction of monthly mortalities due to piscirickettsiosis was observed during the AGD outbreaks. AGD did not seem to affect the prevalence of ISAV or sea lice abundance when chemical bath was used to treat for sea lice. The isolation of the pathogenic strain ISAV 752 and ISAV901 as subclinical infection on salmon farms at the eastern coast of Chiloé

archipelago makes it very risky to apply the stressful routine chemical bath to treat sea lice. These baths could cause more stress to fish which were already immunosuppressed by sea lice and could promote the multiplication of the ectoparasite after treatments and cause outbreaks of other diseases. In case of AGD, fish should not be treated for sea lice with chemical baths because their already injured gills may then trigger fish mortalities.

CHAPTER 6

GENERAL DISCUSSION

To the best of my knowledge, there have been no previous studies considering an integrated overview of ectoparasites and associated pathogens of farmed salmon on seawater sites. The integrated control strategies for the co-infections present on a salmon farm were determined to be a better environmentally friendly alternative than the ineffective control strategies by individual treatments for each disease on several neighbour farms in Chile. Some recommendations that were good for handling one pathogen or parasite were not good in case of another pathogen and these practices should be reviewed and changed.

In the developments of management strategies ectoparasites such as sea lice in salmon aquaculture are mostly considered as reservoirs and vectors of bacterial and viral pathogens (Larenas *et al.*, 1996; Evelyn *et al.*, 1998; Kent, 2000; Birkbeck *et al.*, 2004; Overstreet *et al.*, 2009; Nowak *et al.*, 2010; Oelckers *et al.*, 2014). The results of this thesis showed that the concentration of *Neoparamoeba perurans* was very low or absent in the isopod *C. banksii* indicating that these parasites were unlikely to be important reservoirs or vectors of the pathogenic amoeba in particular, when freshwater treatments were used. In relation to other reservoirs such sea water and inert surfaces, the exploratory study identifying the spatial distribution of *N. perurans* in recirculating water systems holding Atlantic salmon, experimentally inoculated with this pathogen, determined that the amoeba was absent from inert surfaces such as the interphase tank-water-air. The study showed the presence of the pathogen in the water column what was reported before by Bridle *et al.* (2010) identifying *N. perurans* as free living amoebae. Nevertheless, the very low concentration (13 ± 7 cells/L) found in this thesis compared to fish gills where they can reach more than 1000 cells/swab indicates that sea water is not an important reservoir of the pathogenic amoeba; although horizontal transmission of the pathogenic amoeba between hosts could be possible but not through salmon farm gears. This provides some evidence that the salmon could be the main reservoir of *N. perurans* in this experimental system and on farms.

This thesis described the co-infection with *P. salmonis* as one of the possible cause of the chronic presence and upsurge of "caligidosis" due to *C. rogercresseyi* infestation on salmon farms in Chile. As *P. salmonis* was only present in the morbid and moribund fish at Farms 1, 2 and 3 in 2013 and absent in the apparently healthy swimming fish in Atlantic salmon at

Farm 3, presence of this pathogen on sick fish (due to the host immunosuppression) would explain the new outbreaks of sea lice that multiply and spread to healthy fish. In contrast, the presence of the pathogenic strain of ISAV in all of the sampling dates, on moribund or apparently healthy ones, although not in all the fish sampled, indicated that the virus that was present at subclinical levels was not the cause for salmon stress and immunosuppression that allow sea lice multiplication. The other pathology present in all the fish sampled in Chile in this study as a chronic disease was the gill inflammation seasonally caused by AGD. This is the first report of the seasonal appearance of AGD in Chile and of chronic gill diseases. The presence of this gill condition and piscirickettsiosis could be the factors responsible for salmon stress, possible immunosuppression and allow sea lice outbreaks. In this case, repetitive chemical baths to treat these ectoparasites seem inappropriate because of the damage they also cause to fish gills. The routine application of the in-feed treatment for sea lice however, was adequate when emamectin benzoate was still available as effective treatments for the *Caligus* Monitoring and Control program (Zagmutt-Vergara *et al.*, 2005).

Freshwater baths used for AGD by the Tasmanian salmon industry are considered a likely environmentally friendly alternative to the ineffective chemical baths to treat sea lice in Chile although previous report disagree on their utility for *Lepeophtheirus salmonis* (Stone *et al.*, 2002). In this thesis, several gill isopods *Ceratothoa banksii* and some skin isopods *Nerocila orbignyi* were present on farmed Atlantic salmon not treated for AGD by freshwater bath for at least 200 days in summer. However after routine freshwater baths only one juvenile isopod was observed on salmon gills and no adult isopods. Freshwater baths, in addition to reducing the number of amoebae, radically reduce the AGD gill lesions, remove excess mucus and reduce any hypernatraemia (or dehydration) that may develop (Zilberg and Munday, 2006). As the freshwater treatment only mitigates AGD but does not completely eliminate the pathogenic agent, freshwater baths need to be regularly repeated throughout the year. Although an experiment determining the impact of freshwater bathing on isopods was not possible during this research, the relationship between freshwater baths and the lack of isopods is commonly known by Tasmanian salmon farmers (Eduardo Canossa, Senior Technical Officer, Tassal, pers. comm.). The unbathed salmon could be checked only in winter when the water temperature or salinity reduction due to more rainfall did not allow the development of AGD on farmed fish, except for some experiments for selection of AGD resistant salmon strains. In Chile, low salinity water has been proven to reduce the survival of

other ectoparasitic larvae of the sea louse *Caligus rogercresseyi* and allowed adult detachment from host as described below. *In vitro* experiments demonstrated that the eggs of this species could not hatch at salinity 7 psu (González and Carvajal, 1999), larvae could not survive at salinity 15 psu and that survival was low at 20 psu(González and Carvajal, 1999; 2003; Bravo *et al.*, 2008b). Furthermore, no sea lice were found on a farm located in estuaries with salinity 7 psu (González and Carvajal, 1999; González *et al.*, 2000). Zagmutt-Vergara *et al*. (2005) determined that salinity as well as temperature had a linear effect on *C. rogercresseyi* counts where low temperature and salinities were correlated with a lower number of the copepod. This is why some farms in Chile have been deliberately located in estuarine waters to avoid high sea lice burdens (Bravo *et al.*, 2008). However, most farms for salmon grow-out in Chile are located in waters with salinities between 25-33 psu because it is more appropriate for salmon growth but unfortunately more suitable for sea lice reproduction (Zagmutt-Vergara *et al.*, 2005). Given the relationship between sea lice and salinity, freshwater baths such as those used on Tasmanian salmon farms, seem better candidates as commercial and environmentally friendly treatments for ectoparasites normally occurring on most commercial salmon farms in other countries (Johnson *et al.*, 2004). Long term experimental and logistic studies are required to determine the feasibility of freshwater because of the technology used and the need to have freshwater resources near the culture sites. It is noteworthy that in relation to sea lice, a survey in Tasmania (2011) found only five specimens of *Caligus longirostris,* four of which were not reproductive females and a male, after examination of 447 farmed Atlantic salmon affected by AGD but that had not been bathed in freshwater for 236 and 294 days. Therefore, although sea lice were present the disease was not. Despite estuarine location of salmon farms being considered beneficial for the reduction in sea lice, an increase in the density of farms in these areas in Chile was considered a risk of cross-contamination between farms with the other pathogens such as bacteria and viruses (Alvial *et al.*, 2012). This is why the need to decrease the farm densities in estuaries (and in freshwater sites) was suggested in Chile, by increasing for instance, the smolt production in recirculation systems on land (Nieto *et al.*, 2010).

In Chile AGD does not need to be treated (Alvial *et al.*, 2012). The AGD reported in this thesis in this country was a seasonal and mild outbreak and soon disappeared as did in the previously reported outbreaks (Bustos *et al.*, 2011; Rozas *et al.*, 2012); therefore, a freshwater bath treatment seems unnecessary for this condition. In relation to Australia, salmon farms in

Tasmania are located in four distinct estuarine areas. These areas are divided in two zones, one where AGD is absent (West and North Tasmania) and the other where it is present (South East Tasmania)(Douglas-Helders *et al.*, 2005). The environmental factors associated with the clinical AGD disease were high sea water temperatures, high salinities and the retraction of the halocline due to reduction of rain levels and lower freshwater input from the river (Adams and Nowak, 2003). In Chile, the disease was initially described in the East coast of the Chiloé archipelago (Bustos *et al.*, 2011; Rozas *et al.*, 2012) which are all full salinity sites and not close to estuarine areas. Recently, AGD has been identified affecting salmon farms in the XI Region (M. Rozas, pers. comm.) where the density of farms increased after the ISA epidemic (Sernapesca, 2009). In this study, not only high temperature and high salinity were associated with the presence of AGD but also low oxygen concentrations (near 5 mg/L) and fish mortalities when coupled with chemical bath treatments for sea lice. A clear reason for the AGD distribution in Tasmania, Chile or elsewhere is still missing; indicating that more studies related to *N. perurans* ecology are necessary. From the perspective of the model applied by Snieszko (Plumb, 1994), the development of infectious diseases on fish according to the host/pathogen/environment relationship, environmental stress on fish increases when conditions are reaching the tolerance levels of the fish. In this theory, in addition to the co-existence of susceptible host and pathogen, unfavorable environmental conditions must be present for an infectious disease to occur. In the case of Chile this could be due to the chemical bath that adds an environmental negative condition for the hyperplastic and AGD affected gills of fish. This is explained by the model:

$H\ (A + S^2) = D$ where H is the host; A is the etiological agent; S are the environmental stressors; and D is the disease (Plumb, 1994).

In the model, the environmental stressors are squared when host approaches adaptation limits, because detrimental effects are magnified.

Although the frequently method used in Tasmania to prevent and treat for AGD are the routine freshwater baht, the reduction of fish density has also been recommended (Zilberg and Munday, 2006). Investigations of other alternative treatments for AGD have included the oxidative disinfectants such as hydrogen peroxide (Adams *et al.*, 2012; Powell and Kristensen, 2014) that have also been applied to treat sea lice (Bravo *et al.*, 2010). This oxidative disinfectant has several patho-physiological effects on the gill epithelium of

salmonids (Powell and Clark, 2004). These effects include an acute congestion of the gill filament and central venous sinus. The lamellar epithelium is often crenated or denuded, and often associated with epithelial cell necrosis (Powell and Kristensen, 2014). Therefore, such treatments that also increase the gill injury seem inadequate for already damaged gills of farmed salmon in Chile. In relation to immunization measures, at present the development of vaccines for AGD have not been successful (Clark *et al.*, 2003; Valdenegro, 2014; Valdenegro-Vega *et al.*, 2015).

The sanitary crisis in Chilean salmon industry caused by the ISA outbreak began in July 2007 when the salmon industry was most concentrated (Alvial *et al.*, 2012) and contingency measures although appropriate for ISA were not necessary suitable for sea lice pest management. As much as 40% of the total salmon production in this year was in the central and east coasts of Chiloé Island where the epidemic started (Mardones *et al.*, 2011b). However, the virus was thought to be present in the area before 2007 (Kibenge *et al.*, 2009). The start of the epidemic coincided with the large number of farms and the stress caused by high density of fish reaching 25 to 30 kg/m^3 (Mardones *et al.*, 2009), poor quality of smolts produced to meet the required number of fish for grow out and commercialised abroad between 2004 and 2007, sea lice outbreaks and the intensive plan to control *Caligus* was implemented (Sernapesca, 2007b; 2010). In response to the crisis, the Government enacted the Contingency plan to control ISA (between 2007 and 2008). In addition, a protocol of good practices to control ISA and *Caligus* was developed consisting of health measures that were then supported by regulation (government law Resolución 1577 Exenta) to be fully implemented in 2009. The contingency measures added to the protocol of good practices are detailed in Table 6.1 (Alvial *et al.*, 2012).

Table 6.1. Contingency measures and Code of good practices for ISA and *Caligus* undertaken by the government (adapted from Alvial *et al.*, 2013)

1. Cull fish in cages positives for ISAV with pathogenic strains depending on the prevalence (except for infections with HPR0 strain), treat the effluent liquid waste, quarantine infected farms and associated farms until fish are removed
2. Restriction of fish movements
3. Inactivation of pathogens in dead fish
4. All in all out of fish (during a period of 3 months) with fallowing periods and zone management (no treatments are available for the disease). Fallowing periods of 3 months.
5. Biweekly sea lice monitoring and coordination of control when an average abundance of 6 adult lice per fish; improve sea lice management by permitting the use and alternation of more than three drugs for treatments.
6. Reduce handling and use of drug treatments for long term farming
7. Development, implementation and service support for fish health surveillance and diagnostic capacities (hired by Sernapesca but paid by the industry)
8. Single species at all sites.
9. Vaccination of fish (not compulsory at present)
10. Adequate disinfection of eggs and the use of only good quality smolts.
11. Reduction of farm total biomass (from 1.2 million to 800.000), of cage number (to 18-20) and maximum density of 17 kg/m^3.

Although these measures were important for solving the sanitary crisis that occurred, there are some problems that are evident according to the results of this thesis. For instance, point number 5 (control of sea lice on fish when the abundance is over 6 adults per fish) is contrary to point number 6 (reduction of handling and use of drugs) particularly in case of bath chemical treatments for sea lice. An amendment was made to the regulation to help with the reported problem of sea lice reduced sensitivity to emamectin benzoate (Bravo *et al.*, 2008a) after the outbreaks in 2006-2007. This amendment was the approval of other drugs for use to ensure the availability of treatments for sea lice and alternation of drugs to reduce the risk of sea lice resistance. Nevertheless, these drugs that were tested previously and do not have the same efficacy of emamectin benzoate could only be applied in bath (Roth, 2000). In relation to the minimum distance between farms that is part of the integrated management strategy by Sanitary Areas, the regulated distance is 1.5 marine miles or 2.778 km what explains the location between Farms 1 and 2 described in this thesis. This minimum distance is not related with the dispersion of sea lice larvae and can cause easy cross-infections of sea lice between farms (Molinet *et al.*, 2011; Middlemas *et al.*, 2013); this in spite of the reduction in fish biomass per area implemented since 2008 and described in point number 11 of the contingency measures and code of practice (Table 6.1).

Based on the results of previous programs on sea lice and ISA control in Chile, a new survey and control program for piscirickettsiosis was regulated and implemented in December 2012 (Resolución Exenta 3174, Sernapesca, Chile). In this program piscirickettsiosis is considered as a high risk disease although the disease was already described causing mortalities in Chile since 1989. The program aims at a reduction of the disease impact by early detection using epidemiological survey and control. During piscirickettsiosis outbreaks that occur in all of the three species most cultured in Chile (Atlantic salmon, rainbow trout and coho salmon), mortalities of 20 to 30% have been reported although in some cases mortalities can reach 90% (Resolución Exenta 3174, Sernapesca, Chile). Farms with mortalities of 0.35% per week or over in one or more cages are classified as an “Alert Site” and an action plan to reduce mortalities is compulsory. Farms with mortality over this value in 50% of cages or more for 4 weeks are classified as “Sites of High Spread” and if the sanitary action plan of the farm cannot succeed in reducing the mortality within a period of 6 weeks, the early harvesting of the problematic cages are mandatory. For the survey, a sampling and PCR diagnosis for *P. salmonis* is required every two months on every farm where fish show pathological signs of

the disease, are moribund or rejected fish and experience mortality due to unspecific causes. As husbandry measures for piscirickettsiosis mortality, the action plan includes increase in the removal of dead fish in the affected cages to twice daily. According to the regulation, the plan could include the removal of moribund fish, dividing the fish population from one problematic cage into the two cages, harvest or discarding of the problematic cages and treatments of affected cages with antibiotics. Additionally the implementation of prophylactic measures such as use of vaccines, immunostimulant compounds applied in feed and reducing the stock density of fish are recommended by the piscirickettsiosis plan. According to the results in this thesis, it would be important not only to remove the dead fish but also the moribund fish that are the reservoirs of the pathogen. Also, the commercial antibiotics that have shown lack of efficiency on farms (Rozas and Enríquez, 2014) would probably not be a solution and a risk of other bacteria resistance. In relation to vaccines, they can cause adhesions as a side effect and the mortality of fish by digestive tract constriction (Bruno *et al.*, 2013). Adhesions were in fact the first cause of death in the accumulated mortalities in the whole salmon growing cycle at Farm 2 in this thesis, indicating more fish risk with vaccines.

CONCLUSION

Considering an integrated overview of the impact of the main parasitic diseases on farmed salmon, associated co-infections and control strategies in Australia compared to Chile, the following results were found:

1) Gill isopods *Ceratothoa banksii* and skin isopods *Nerocila orbignyi* were found affecting farmed Atlantic salmon in Tasmania when fish were experimentally not bathed to treat AGD for more than 7 months. Although the copepod *Caligus longirostris* was present, it was rare on those fish and in very low prevalence (1-2%) in summer samplings.
2) Although *C. banksii* can contain *N. perurans* on its body surface in very low concentration it is unlikely that these isopods could act as a significant vector of the amoeba and transmit the pathogen from one parasitised fish to another host. This risk is further minimised by freshwater bathing which removes the isopods.

3) The amoeba reached a very low concentration in sea water (13 ± 11 cells/L) and was absent from inert structures such as tank surfaces. Morbid farmed fish still remain the most important reservoir for *N. perurans*.
4) This is the first report of a seasonal appearance of AGD on farmed salmon in Chile and chronic gill damage sometimes related with AGD, but not culture-born contaminants such as ammonia, diatoms, dinoflagellates or copper. Chronic gill lesions indicate a probable primary cause for sea lice outbreaks and the inappropriate use of gill injuring chemical bath treatments for the control of the parasites on farms.
5) Low oxygen concentration on a sea farm site could be a risky environmental condition for fish mortalities associated with the seasonal appearance of AGD in Chile, in addition to high sea water temperature and salinity.
6) The most prevalent co-infection after sea lice was *Piscirickettsia salmonis*. The presence/absence of AGD did not affect the proportion of ISAV positive fish or sea lice abundance on farmed fish when chemical bath treatments were applied, although some differences were observed in monthly mortalities due to *P. salmonis*.
7) The isolated of the pathogenic strains ISAV 752 and ISAV901 from fish farmed in Chile were at subclinical infection levels when fish were not chemically bathed for sea lice. The presence of these strains in infected fish makes it very dangerous to apply the stressing routine chemical bath to treat sea lice.

The integrated control strategies for the co-infections present on each salmon farm was determined to be a better alternative to develop environmentally friendly pathogen and pest management strategies in Chile than the ineffective integrated and coordinated control strategies for a single pathogen in several neighbour farms.

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